The characterisation of *ex vivo* generated Epstein-Barr virus-specific cytotoxic T-cell lines

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Declaration of authorship

This thesis was composed by the undersigned candidate. The work included is the candidate's own, with contributions from other researchers, unless otherwise stated.

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Abstract

Post-transplant lymphoproliferative disease (PTLD) is an Epstein-Barr virus (EBV)-associated disease, which occurs in up to 10% of transplant patients and can be fatal in up to 70% of cases. PTLD has been successfully treated with cellular adoptive immunotherapy using EBV-specific cytotoxic T-cell lines (CTLs). However, little is known about the development of these CTLs. This project therefore sought to establish how the CTLs developed in terms of their growth, phenotype and T-cell receptor (TCR) repertoire diversity. It also sought to establish which cytolytic molecules were present in the CTLs and which pathways they employed to effect their cytotoxic potential.

The phenotype and the cytolytic proteins of the CTLs were investigated by fluorescence activated cell sorting and western blotting. The TCR repertoire diversity was established by spectratyping analysis of the complementarity determining region 3 of the variable β chain of the TCR. Cytotoxic activity and pathways were assessed using chromium release assays.

The results show that the CTLs developed phenotypically into mature T-cells within the first four weeks of culture, with an increase in CD45RO and CD69 expression and a decrease in CD45RA, CD62L, CD27 and CD28 expression. The CTLs also expanded most during that period and had viability in excess of 60%. The growth and viability of the polyclonal CTLs decreased continuously after four weeks of culture. The TCR repertoire of the CTLs remained diverse during the course of culture. Granzyme B, perforin and Fas ligand (FasL) were all detected in the CTLs. There was significantly more granzyme B in CD8+ T-cells than in CD4+ T-cells ($p=0.0016$) and there was also a significantly greater proportion of CD8+ T-cells expressing granzyme B than CD4+ T-cells ($p=0.0023$). There was no significant difference in the proportion of CD4+ T-cells and CD8+ T-cells expressing FasL or perforin, or in the amounts of FasL or perforin found in the respective cell types. Granulysin was detected in 33% of the CTLs tested. Cytotoxicity was significantly

inhibited by concanamycin A ($p=0.0007$) and ethylene glycol-bis tetraacetic acid ($p<0.0001$) indicating that the principal cytotoxic pathway employed by the CTLs was a calcium and perforin-mediated exocytosis pathway leading to the release of granzyme B.

These findings suggest that CTLs develop into mature CTLs in the early stages of culture without a reduction in TCR repertoire diversity. These findings also suggest that both CD4+ T-cells and CD8+ T-cells contribute to the overall cytotoxicity of the CTLs using a perforin-mediated cytotoxic granule exocytosis pathway. It is hoped that this basic characterisation of CTLs will reveal features that can potentially enhance the clinical effects of CTLs.

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This thesis is dedicated to my Grandparents – Trudi and Louis.
Cette thèse est dediée à mes Grandparents – Trudi et Louis.

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Abbreviations and symbols used

Abbreviations

A	Absorbance
AIDS	Acquired immunodeficiency disease syndrome
BART	Bam A rightward transcripts
BFA	Brefeldin A
BL	Burkitt's lymphoma
BMLF	Bam M leftward open reading frame
BMT	Bone marrow transplant
bp	Base pair
Bq	Becquerel
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine
Ca	Calcium
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CMA	Concanamycin A
CO_2	Carbon dioxide
Cr	Chromium
Cs	Caesium
CsA	Cyclosporin A
CTL	Cytotoxic T-cell line
Da	Dalton
dATP	Deoxyadenosine 5' triphosphate
DC	Dendritic cell
dCTP	Deoxycytosine 5' triphosphate
dGTP	Deoxyguanine 5' triphosphate
dH_2O	Distilled water
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethyl sulphomethoxasol
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5' triphosphate
dTTP	Deoxythymidine 5' triphosphate
DTT	Dithiothreitol
EBER	Epstein-Barr virus encoded small RNAs
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	Ethylene diamine-tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid
E:T	Effector:target ratio
FACS	Fluorescence associated cell sorting
Fab	Antibody fragment
FAM	Carboxyfluorescein

FasL	Fas ligand
FBS	Foetal bovine serum
Fc	Crystalline fragment
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
g	Gram
G	Giga
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	Glycoprotein
GrB	Granzyme B
GVHD	Graft versus host disease
HBSS	Hank's balanced salt solution
H_2O	Water
HCl	Hydrochloric acid
HCMV/CMV	Human cytomegalovirus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human leucocyte antigen
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
k	Kilo
KSHV	Kaposi sarcoma associated herpes virus
l	Litre
LCL	Lymphoblastoid cell line
L-Glut	L-Glutamine
LMP	Latent membrane protein
LP	Leader protein
m	Milli
M	Molar (moles/l)
MA	Membrane antigen
MFI	Mean fluorescence intensity
Mg	Magnesium
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
NA	Not available
N/A	Not applicable
NBF	Normal buffered formaline
NEAA	Non-essential amino acids

NFH_2O	Nuclease-free water
NK	Natural killer
NPC	Nasopharyngeal carcinoma
OHL	Oral hairy leucoplakia
ORF	Open reading frame
p	Pico (10^{-12})
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PECy5	Phycoerythrin Cyanin 5
Per	Perforin
P/S	Pencillin/Steptomycin
PTLD	Post-transplant lymphoproliferative disease
RNA	Ribonucleic acid
RPMI	Rosewell Park Memorial Institute
RT-PCR	Reverse transcriptase-polymerase chain reaction
s	Second
S	Weekly stimulation with LCL
SAP	Signalling lymphocytic-activation molecule (SLAM) associated protein
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SLAM	Signalling lymphocytic-activation molecule
SNBTS	Scottish National Blood Transfusion Services
SPBS	Sterile phosphate buffered saline
TBE	Tris borate EDTA
TCR	T-cell receptor
Th	T helper
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
UI	International unit/ Unité internationale
Vβ	Variable β chain
VHAS	Virus-associated haemophagocytic syndrom
VCA	Viral capsid antigen
v/v	Volume/volume
VZV	Varicella zoster virus
w/v	Weight/volume
X-LPS	X-linked lymphoproliferative syndrome

Symbols used

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
μ	Micro
%	Percentage
$\approx$	Approximately
°C	Degree Celcius
1°	Primary
2°	Secondary
g	g number

Chapter 1

Introduction

1.1 Herpesviridae

1.2 Epstein-Barr virus

1.3 Epstein-Barr virus: immune responses and associated diseases

1.4 Post-transplant lymphoproliferative disease

1.5 Adoptive immunotherapy

1.6 Aim

1.1 Herpesviridae

Herpes viruses are the most widely disseminated viruses, with over 130 known herpes viruses infecting mammals and birds, but also reptiles such as turtles and invertebrates such as oysters (Farley *et al*, 1972; Pass, 2003). Herpes viruses are divided into three subfamilies – *Alpha-*, *Beta-* and *Gammaherpesvirinae*. These three subfamilies are thought to have arisen between 220 to 180 millions years ago and that sublineages or genera, within these subfamilies in turn arose 80 to 60 millions years ago (McGeoch *et al*, 1995). A new class of herpesviruses was recently identified in Pacific oysters (Davison *et al*, 2005).

1.1.1 Herpes virus structure

Herpes viruses are double-stranded enveloped DNA viruses with co-linear genomes ranging in size from 125 to 230 kilobase pairs (kbp) and encoding between 70 to 200 genes (Roizmann *et al*, 1992). Herpes viruses contain a toroid-shaped protein core surrounded by DNA and an icosadeltahedral nucleocapsid with 162 capsomeres in a T=16 arrangement (Kieff and Rickinson, 2001). The diameter of the nucleocapsid ranges from 100 to 110 nm (Cleator and Klapper, 2000). There is an amorphous protein tegument between the nucleocapsid and the glycoprotein-bearing envelope (Kieff and Rickinson, 2001). Typically, the diameter of human herpes virus (HHV) virions ranges between 110nm and 300nm (Roizman and Baines, 1991). Figure 1.1 diagrammatically represents the structure of human cytomegalovirus (HCMV) and illustrates the principal components of the virus. This basic structure is common to all herpes viruses.

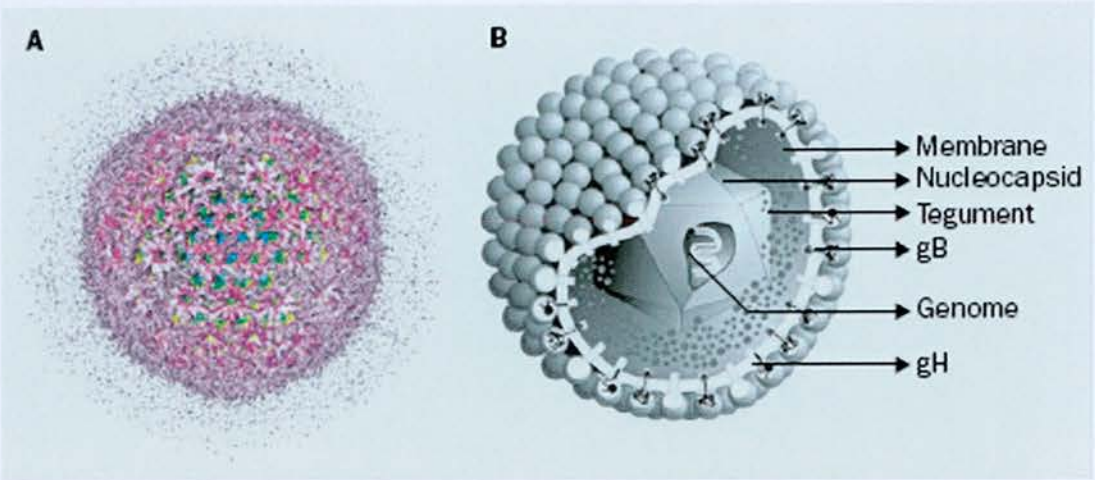


Figure 1.1: Human cytomegalovirus. A) Diagram of the virus. B). Virtual 3-dimensional model showing viral component (gB: glycoprotein B and gH: glycoprotein H) (Gandhi and Khanna, 2004). **A) Reprinted from Virology, Volume 260, Chen DH, Jiang H, Lee M, Liu F and Zhou ZH, Three-dimensional visualisation of tegument/capsid interaction in the intact human cytomegalovirus, page no 10-16, Copyright (1999), with permission from Elsevier. B) Reproduced with permission from Marko Reschke, www.biografix.de.**

1.1.2 Human Herpes Viruses

Eight HHVs have been identified to date, HHV-8 being the last HHV to have been identified by Chang *et al* in 1994 (Chang *et al*, 1994), and these are listed in table 1.1. The genus that each HHV belongs to is also given in table 1.1. The Cercopithecine herpesvirus 1, a herpes virus of Old World monkeys, can infect and be fatal to humans following bites from an infected animal (Whitley, 2001). This virus is not listed in table 1.1, as its natural host is Old World monkeys and it is therefore not a true HHV. Cercopithecine herpesvirus 1 was fully sequenced recently by Tyler *et al* (Tyler *et al*, 2005).

Official name	Subfamily	Genus (*-virus*)	Common name and abbreviation
HHV-1	*Alpha-*	*Simplex-*	Herpes Simplex-1 (HSV-1)
HHV-2	*Alpha-*	*Simplex-*	Herpes Simplex-2 (HSV-2)
HHV-3	*Alpha-*	*Varicello-*	Varicella Zoster Virus (VZV)
HHV-4	*Gamma-*	*Lymphocrypto-*	Epstein-Barr Virus (EBV)
HHV-5	*Beta-*	*Cytomegalo-*	Human Cytomegalovirus (HCMV)
HHV-6	*Beta-*	*Roseolo-*	Human Herpesvirus-6 (HHV-6)
HHV-7	*Beta-*	*Roseolo-*	Human Herpesvirus-7 (HHV-7)
HHV-8	*Gamma-*	*Rhadino-*	Kaposi's Sarcoma-associated Herpes Virus (KSHV)

Table 1.1: Known human herpes viruses – compiled using information from Cleator and Klapper (2000) and (Anonymous, 2001).

HHVs are categorised into the *alpha-*, *beta-* and *gamma*-herpesviruses subfamilies according to their biological properties, and they are further classified into genera by deoxyribonucleic acid (DNA) sequence homology and genome arrangement, as well as homology between major viral proteins (Roizman and Baines, 1991). The four principal biological properties of herpes viruses are summarised below (Roizmann *et al*, 1992) and the biological properties that define each subfamily are given in table 1.2.

1. Herpes viruses encode genes for enzymes involved in nucleic acid metabolism and DNA synthesis. Enzymes involved in protein processing are also sometimes encoded. The number and nature of these enzymes varies depending on the herpes virus in question.

2. Viral DNA synthesis and capsid assembly occurs in the nucleus and the capsid is enveloped as it transits through the nuclear membrane.

3. Progeny virus production is always associated with cell death during lytic replication.

4. Known herpes viruses are all able to remain latent in their natural host. During latency the viral genome circularises into an episome. The expression of viral genes in latently infected cells is restricted thus enabling the virus to persist.

Subfamily	**Genus (*-virus*)**	**Biological properties**		
		Growth cycle	**Cytopathology**	**Site of latency**
Alpha	*Simplex-*	Short	Cytolytic	Sensory ganglia Neurons
	Varicello-			
Beta	*Cytomegalo-*	Long	Cytomegalic	Glands (adrenal, salivary) and lymphocytes
				Kidneys
	Roseolo-	Long	Lymphoproliferative	Lymphoid tissue
Gamma	*Lymphocrypto-*	Variable	Lymphoproliferative	Lymphocytes
	Rhadino-			

Table 1.2: Biological properties of human herpes subfamilies and genera. Adapted from Whitley (2001).

1.2 Epstein-Barr Virus

Dennis Burkitt, a British surgeon, while working in East Africa recognised and defined a previously unknown childhood tumour in 1958, which is now known as Burkitt's lymphoma (BL) (Burkitt, 1958). The unusual epidemiology of BL across equatorial Africa, where malaria is endemic, led Burkitt to postulate that the tumour he recognised was of infectious aetiology and that mosquitoes were the carriers of this infectious agent (Burkitt, 1962). In 1961, Anthony Epstein met Dennis Burkitt following a talk by the British surgeon on BL and it was agreed that tumour material would be shipped from East Africa to the Middlesex Hospital in London where Epstein's laboratory was established. Initial work using electron microscopy and standard virological methods was unsuccessful but the generation and the examination by electron microscopy of the first *in vitro* BL cell line yielded positive results with the detection of herpes virus-like particles (Epstein, 1979; Epstein *et al*, 1964; Epstein and Barr, 1964). The biological and immunological properties of these particles were assessed and confirmed that the particles were a new herpes virus known as EBV (Epstein *et al*, 1965). EBV is now known to infect B-cells, T-cells and epithelial cell types, as well as other cells, both *in vitro* and *in vivo*. It is now also known to be associated with a number of diseases, such as infectious mononucleosis, all of which will be presented in section 1.3.2. The molecular biology of EBV is discussed in this section and its epidemiology is discussed in section 1.3.

1.2.1 EBV genome, proteins and latency programmes

1.2.1.1 EBV genome

The genome of the EBV B95-8 prototype laboratory strain was first sequenced and cloned in 1984 (Baer *et al*, 1984). It is 172 kb in length and contains 84 open reading frames (ORFs). EBV was sequenced from an EBV DNA *Bam*HI fragment cloned library and therefore ORFs, genes or sites for transcription of ribonucleic acid are often referred to as specific *Bam*HI DNA fragments. So, the gene encoding EBV

DNA polymerase is referred to as *BALF3* – *Bam*H1 fragment A, third leftward ORF (Kieff and Rickinson, 2001). The B95-8 prototype laboratory strain of EBV was first isolated by Miller *et al* in 1973 (Miller *et al*, 1973). A *Bam*HI digest of the EBV B95-8 genome is shown in figure 1.2. The ORFs encoding EBV latent proteins – Epstein-Barr Nuclear Antigens (EBNAs) and Latent Membrane Proteins (LMPs) are shown in figure 1.2. Latent membrane protein 2 (LMP-2), not shown in this diagram, originates from messenger RNA (mRNA) transcripts that are spliced across the terminal repeats (TR) of the circularised genome (Young and Murray, 2003). The circularised genome is shown in figure 1.3. The role of each of these proteins will be discussed in section 1.2.1.2.

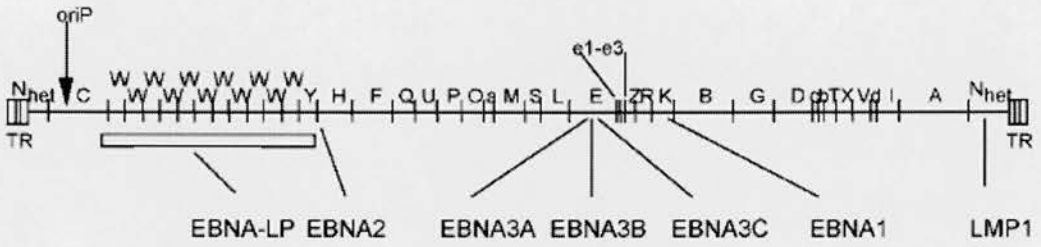


Figure 1.2: *Bam*HI digest of the EBV B95-8 genome (showing the ORFs of EBV latent proteins). The fragments are named according to their size with A being the largest and smaller fragments being identified by lower case letters. EBNA-LP: Epstein-Barr Nuclear Antigen-Leader Protein. **Reprinted by permission from Macmillan Publishers Ltd: Young LS and Murray PG. Oncogene 22: 5108-512, copyright (2003).**

1.2.1.2 EBV encoded transcripts and proteins

EBV has the ability to express different combinations of genes and therefore different proteins, depending on whether the infection is latent or lytic. EBV can achieve this by using different gene promoters. Latent and lytic infections will be described in subsequent sections of this chapter.

a) EBV latent replication and proteins

During latency EBV can express up to nine latent viral antigens, six of which are nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-Leader Protein (LP)) and three of which are membrane proteins (LMP-1, LMP-2A and LMP-2B). Epstein-Barr virus encoded small RNAs (EBER1 and EBER2) are also transcribed, but not translated, during latency. Transcripts from the *Bam*HI A region are also detected and these have been implicated in growth regulation in some cancers such as nasopharyngeal carcinoma (NPC) (Smith *et al*, 2000). All of the latent genes are shown in figure 1.3 which represents the episomal genome of EBV.

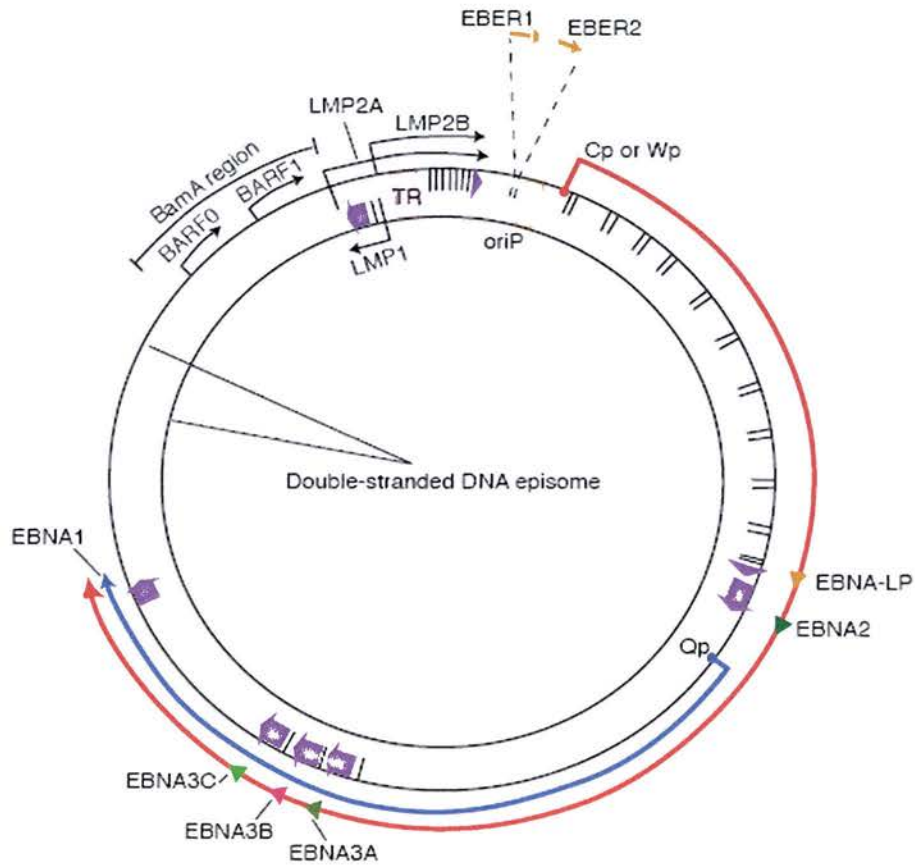


Figure 1.3: EBV episomal genome. The genes encoding the latent proteins, marked by purple arrows, are also shown in this figure. The origin of plasmid replication is *oriP*. The promoters involved in the transcription of the message encoding these proteins are also shown, promoter C (Cp), or Wp and Qp. Diagram from Murray and Young (2001). **Reprinted by permission from Cambridge University Press. Murray PG and Young LS. Expert Reviews in Molecular Medicine 2001: 1-20, Copyright.**

In a typical EBV infection of human B-cells *in vitro*, the EBV linear genome circularises into its episomal form 24 hours post-infection (Hurley and Thorley-Lawson, 1988), although Alfieri *et al* (1991) detected episomes as early as 12 hours post-infection. All EBNAs arise from a single mRNA transcript that is differentially spliced and under the control of the Cp and Wp promoters. The Wp promoter is preferentially used in the initial stages of the infection, whilst the Cp promoter is normally used in the latent infection and their use is mutually exclusive (Woisetschlaeger *et al*, 1989; Woisetschlaeger *et al*, 1990). The LMP mRNA transcripts are expressed under the control of promoters found in the *Bam*HI N region (Young and Murray, 2003). EBNA-2 and EBNA-LP are the first genes to be expressed and their products can be detected between 8 and 12 hours post-infection (Alfieri *et al*, 1991; Allday *et al*, 1989; Rooney *et al*, 1989; Wang *et al*, 1990b). EBNA-2 is expressed under the control of the Wp promoter, which then leads to a promoter switch to the Cp promoter (Woisetschlaeger *et al*, 1991). The switch in promoter usage occurs approximately 36 hours post-infection, which coincides with the expression of EBNA-1, EBNA-3A, B and C, although in one study these EBNAs were detected sooner (12-18 hours post-infection) (Allday *et al*, 1989). LMP-1 is expressed in parallel with or subsequent to EBNA-1 expression, as EBNA-1 acts as a transcriptional transactivator upregulating the LMP-1 promoter (Rickinson and Kieff, 2001). LMP-2A and -2B are not detected for up to 70 hours post-infection. This pattern of gene expression, in which all nine latent proteins are expressed, is known as "latency III". This latency programme, as well as latency programmes I and II, will be discussed in section 1.2.1.2 b).

Concomitant with EBV antigen expression, there is upregulation of cellular gene expression encoding cluster of differentiation (CD) 21, CD23, CD39 and CD40 (Cordier *et al*, 1990; Kieff and Rickinson, 2001; Wang *et al*, 1990a). Cellular adhesion markers such as leucocyte-function antigen-1 (LFA-1 or CD11a), LFA-3 (or CD58) and intercellular adhesion molecule-1 (ICAM-1 or CD54) are also upregulated upon EBV infection (Wang *et al*, 1990a). EBV also drives resting B-cells into the G1-phase, from the G0-phase, and then into the S-phase of the cell's

lifecycle, in which the viral genome is replicated only once, concurrently with the B-cell genome, as EBV, like all other herpes viruses is dependent on the host cell machinery for its replication (Adams, 1987; Murray and Young, 2001). All these upregulatory/stimulatory events are co-ordinated by EBV antigens. The role of each antigen is summarised below and is reviewed in Bornkamm and Hammerschmidt (2001), Crawford (2001), Murray and Young (2001) and Young and Murray (2003) unless otherwise stated.

EBNA 1

Reedman & Klein first identified an EBNA in 1973, now known as EBNA-1 (Reedman and Klein, 1973). EBNA-1 expression is initiated by the Cp or Wp promoter in latency III, but in latency I and II it is transcribed from the Qp promoter. EBNA-1 acts as a transcriptional transactivator upregulating the Cp and LMP-1 promoters. The primary role of EBNA-1 is to maintain the episomal genome, which it does by binding to the EBV origin of replication, which has two clusters of EBNA-1 binding sites (Rawlins *et al*, 1985). In order to achieve this, EBNA-1 has glycine-alanine repeats that inhibits its proteosomal degradation and therefore antigen processing and expression by major histocompatibility class I (MHC I), a fact which coincidently increases its half-life (Levitskaya *et al*, 1995; Levitskaya *et al*, 1997). The glycine-alanine repeat has also been found to prevent translation of EBNA-1 mRNA (Yin *et al*, 2003).

EBNA-2

The P-H virus (now known as P3HR1) is unable to immortalise B-cells (Miller *et al*, 1974; Rabson *et al*, 1982) because it lacks the EBNA-2 gene, as well as the last 2 exons of the EBNA-LP gene (Hammerschmidt and Sugden, 1989). This suggests that EBNA-2 is essential for B-cell immortalisation. It regulates the expression of all other latent viral genes, such as LMP-1 and LMP-2, but also transactivates cellular genes controlling the expression of cell surface markers such as CD21 and CD23 (Wang *et al*, 1987; Wang *et al*, 1990b). EBNA-2 interacts with the repression domain of cp-binding protein factor-1 thus converting it to a transcriptional activator.

In that respect, EBNA-2 acts in the same fashion as NotchIC (part of the Notch pathway), which influences cell proliferation and differentiation (Hayward, 2004). EBNA-2 also controls the switch from the Wp promoter to the Cp promoter in early B-cell infection, which leads to the transcription of EBNAs-1, -3A, -3B and -3C and LMP-1. EBNA-2 exercises a pivotal role in EBV immortalisation.

EBNA-3

EBNA-3A, 3B and 3C are transcribed from the Cp promoter. EBNA-3A and -3C are essential for B-cell immortalisation, whereas EBNA-3B is not. EBNA-3B is involved in the regulation of the transcription of cellular genes encoding CD40 (Silins and Sculley, 1994), whilst EBNA-3C regulates viral LMP-1 and cellular CD23 (Allday and Farrell, 1994). EBNA-3C interferes with at least two cell cycle checkpoints at G1 and G2/M phases (Parker *et al*, 2000). Additionally, EBNA-3C can also repress the Cp promoter, which is the major promoter controlling EBNA expression, regardless of EBNA-2 activity (Radkov *et al*, 1997). Collectively, the EBNA-3s are able to interact or interfere with EBNA-2 signalling but are also able to modulate both viral and cellular promoter function.

EBNA-LP

EBNA-LP is not required for B-cell transformation/immortalisation but it promotes the efficient outgrowth of lymphoblastoid cell lines (LCLs) *in vitro* (Allan *et al*, 1992; Hammerschmidt and Sugden, 1989). It interacts closely with EBNA-2 in transcriptional activation and potentiates EBNA-2's transactivating functions, such as the expression of LMP-1 (Nitsche *et al*, 1997). A number of roles have been put forward for EBNA-LP, including cell cycle control and interaction with the tumour repressors p53 and retinoblastoma, but to date none has been confirmed.

LMP-1

LMP-1 is essential for *in vitro* proliferation of B-cells and also for transformation of B-cells therefore acting as an oncogene (Dirmeier *et al*, 2005). It can also induce the expression of a number of cell surface molecules such as CD23, CD39, CD40, CD54

(ICAM-1) and CD58 (LFA-3) in a variety of human B-lymphocytes cell lines (Wang *et al*, 1990a). LMP-1 can also induce the upregulation of the anti-apoptotic molecule Bcl-2 and the production of IL-6 and IL-8. Pro-apoptotic molecules such as Bax can on the other hand be downregulated by LMP-1 (Dirmeier *et al*, 2005). LMP-1 is a member of the tumour-necrosis receptor superfamily, which also activates a number of downstream cellular pathways such as the NF-kB pathway, the JNK/AP-1 pathway, the p38/MAPK and the JAK/STAT pathway. As well as affecting cellular pathways, LMP-1 functionally resembles CD40, which is responsible for B-cell activation, and has been shown to be capable of replacing CD40 *in vivo* providing proliferation and differentiation stimuli to B-cells. In conjunction with its signalling properties LMP-1 has been shown to bind to BZLF-1 (BZLF-1 is a transactivator that controls EBV lytic infection, discussed in section 1.2.1.2 c)) in cells that did not express EBNA-2 (latency programme II discussed in section 1.2.1.2 b)), therefore contributing to the maintenance of latency *in vivo* (Adler *et al*, 2002). CD40 was also shown to bind to BZLF-1 and inhibit reactivation (Adler *et al*, 2002), highlighting the extensive mimicry achieved by LMP-1. Whilst there are functional similarities between LMP-1 and CD40, their signalling methods are quite distinct with LMP-1 involving tumour necrosis receptor-associated death domain (TRAAD) and CD40 involving tumour necrosis receptor-associated factor 6 (TRAF-6).

LMP-2

The LMP-2 gene encodes two distinct proteins LMP-2A and LMP-2B (a truncated form of LMP-2A). The EBV genome is required to circularise for successful transcription of both LMP-2A and LMP-2B. The promoters of both LMP-2A and LMP-2B are under the control of EBNA-2, but LMP-2A can be expressed *in vivo* in memory B-cells in the absence of EBNA-2, suggesting that cellular factors may take over from EBNA-2. Neither LMP-2A nor LMP-2B is essential for B-cell immortalisation *in vitro*, however LMP-2A may be involved in epithelial cell transformation. LMP-2A disrupts B-cell signalling but in turn it acts as a constitutively activated B-cell receptor homologue (Caldwell *et al*, 1998) and it also appears to prevent EBV-infected cells from entering the lytic cycle. These functions

are mediated by the N-terminal of LMP-2A that contains eight tyrosine residues. Two of the tyrosine residue form immunoreceptor tyrosine-based activation motifs, which play key roles in modulating cell proliferation and activation mentioned above. The role of LMP-2B has yet to be established but it is thought that it may be to dampen the effects of LMP-2A.

EBERs

EBERs-1 and -2 are small nonpolyadenylated RNAs, which are transcribed but not translated. They are therefore not EBV antigens *per se* but have been found in all forms of EBV latency (Rickinson and Kieff, 2001). They are the most abundant EBV RNAs. They are not required for immortalisation, however they may have a role in tumourigenesis. Their reintroduction into an EBV-negative Akata BL cell line restored the tumourigenicity in a severe combined immunodeficiency (SCID) mouse model and conferred resistance to apoptosis induction (Komano *et al*, 1999). Additionally, EBERs may induce interleukin-10 production in EBV positive BL cell lines, enhancing their differentiation in autocrine fashion (Kitagawa *et al*, 2000).

Bam H1 A rightward transcripts (BARTs)

Much like EBERs, BARTs are not EBV viral antigens *per se* but highly spliced polyadenylated RNAs, which contain three ORFs (Brooks *et al*, 1993; Hayward, 2004). The function of BARTs remains unclear but they have been identified in a number of malignancies such as undifferentiated nasopharyngeal carcinoma (UNPC/NPC), BL and Hodgkin's disease (HD) and nasal T-cell lymphoma (Chiang *et al*, 1996; Deacon *et al*, 1993; Tao *et al*, 1998). However, the products of two ORFs, RPMS-1 and RK-BARF0, can interact with the Notch pathway (Hayward, 2004) and subsequent expression of LMP-1 (in the case of RPMS-1) (Smith, 2001). The Notch pathway has been implicated in cellular differentiation and proliferation and therefore interactions with this pathway may contribute to the development of EBV malignancies (Hayward, 2004). BARTs have also been found in EBV infected cells from healthy individuals (Chen *et al*, 1999).

The size and cellular location of EBV latent transcripts described is summarised in table 1.3. This table also indicates which transcripts are required for immortalisation.

Protein	Open-reading frame	Molecular weight (kDa)	Cellular site	Required for immortalisation
EBNA-1	BKRF-1	65-97	Nucleus	Yes
EBNA-2	BYRF-1	75-105	Nucleus	Yes
EBNA-3A	BLFR-3/BERF-1	130-195	Nucleus	Yes
EBNA-3B	BERF-2A/B	145-160	Nucleus	No
EBNA-3C	BERF-3/4	130-195	Nucleus	Yes
EBNA-LP	BWRF-1	20-130	Nucleus	No
LMP-1	BNLF-1	58-63	Membrane	Yes
LMP-2A	BARF-1/BNRF-1	54	Membrane	No
LMP-2B	BNFR-1	40	Membrane	No
EBERs	BCRF-1	-	Nucleus/ cytoplasm	No
BARTs	BARF-0	-	Cytoplasm	Not known

Table 1.3: EBV latent transcripts (Johannessen and Crawford, 1999). kDa: kilo Dalton.

b) EBV latency programmes

EBV antigens described above are differentially expressed in a variety of diseases, which will be discussed in section 1.3. The patterns of differential expression are known as latency programmes. There are three latency programmes, as well as the antigen expression seen in B-cells *in vivo* in healthy seropositive individuals and these are summarised in table 1.4.

Latency programme	Antigen(s) expressed	Associated diseases
Normal seropositive individual/ Latency 0	EBERs ** EBNA-1* LMP-2A** BARTs**	Infectious mononucleosis
Latency I / latency programme	EBERs ** EBNA-1 BARTs **	BL
Latency II / default programme	EBERs ** EBNA-1 LMP-1 and/or LMP-2A BARTs **	Reed-Sternberg cells in HD Nasopharyngeal carcinoma (NPC) Undifferentiated NPC (UNPC) T-cell lymphomas
Latency III / growth programme	EBERs ** EBNA-1 EBNA-2 EBNA-3A, -3B, -3C LMP-1, -2A, -2B EBNA-LP BARTs **	Lymphoblastoid cell lines (LCLs) *in vitro* Infectious mononucleosis PTLD

Table 1.4: EBV latency programmes, antigen expression and associated diseases. This table was compiled using information from Callan (2004), Crawford (2001), Hopwood and Crawford (2000) and Thorley-Lawson and Gross (2004). (Callan, 2004; Crawford, 2001; Hopwood and Crawford, 2000; Thorley-Lawson and Gross, 2004). *EBNA-1 is not always detected in dividing cells in healthy seropositive individuals. **only RNA detected.

c) EBV lytic replication

Briefly, EBV is also capable of lytic replication where the EBV genome is amplified 100- to 1000-fold and this replication originates from the origin of replication *oriLyt* (Hammerschmidt and Sugden, 1988). Latent replication and lytic replication are mutually exclusive within one EBV-infected cell. During lytic replication EBV becomes more reliant on its own proteins, particularly DNA polymerase, to sustain its replication (Tsurumi *et al*, 2005). Lytic replication is controlled by an entirely different set of genes to those involved in latent replication. The gene product of the BZLF-1 gene is the control switch between the latent and the lytic state of replication

(Hammerschmidt and Sugden, 1988). The BZLF-1 gene product is a pleiotropic protein that acts as a transactivator setting off the remainder of the lytic gene expression as well as binding to the *oriLyt* and inducing viral replication (Tsurumi *et al*, 2005). Another transactivator is BRLF-1, which acts in concert with BZLF-1, to induce and activate both cellular and viral genes. Both BRLF-1 and BZLF-1 are known as "immediate early" genes and BRLF-1 serves to activate viral DNA polymerase (Hammerschmidt and Sugden, 1988; Liu *et al*, 1996). Thereafter "early genes" are expressed and encode enzymes involved genome replication. Finally, "late genes" are expressed and these encode structural proteins among them glycoprotein 350 (gp350) and gp85. The role of these glycoproteins is discussed in section 1.2.2.2.

1.2.2 EBV infection

1.2.2.1 Cells infected by EBV

EBV has been found to infect a number of different cell types *in vivo* and *in vitro*. It primarily infects B-lymphocytes but also T-lymphocytes and natural killer cells (NK cells) (Kieff and Rickinson, 2001; Pattengale *et al*, 1973). EBV has also been detected in orpharyngeal (squamous) epithelial cells (Sixbey *et al*, 1984) and gastric epithelium (cuboidal/columnar) (Shibata and Weiss, 1992). EBV has also been detected in saliva and salivary glands (Wolf *et al*, 1984), although a recent publication by Frangou *et al* (2005) indicated that salivary glands were not an EBV reservoir. Genital secretions from both men and women have also tested positive for EBV (Israele *et al*, 1991; Sixbey *et al*, 1986). EBV in genital secretions is mostly B-cell-associated (Thomas *et al*, 2006).

1.2.2.2 EBV infection of B-cell *in vitro*

As mentioned above, EBV was found to infect B-cells and its transforming capacity was established following *in vitro* transformation of B-cells and the generation of tumours in nonhuman primates (Henle *et al*, 1967; Miller, 1974; Pope *et al*, 1968). *In vitro* transformed B-cells are referred to as lymphoblastoid cells (LCLs). An

experimentally invaluable method of generating LCLs from infected individuals was developed by Bird *et al* in 1981; the T-cell immunosuppressive agent, cyclosporin A (CsA), was added to peripheral blood mononuclear cells (PBMCs) preventing cytotoxic T-cell activity and allowing outgrowth of LCL (from EBV released from EBV-infected B-cells and infecting other B-cells present in PBMC) (Bird *et al*, 1981; Rickinson, 1984)

EBV enters B-cells *in vitro* by binding to the C3d complement receptor of B-cells using its envelope glycoproteins (Babcock *et al*, 1998; Nemerow *et al*, 1985). The glycoproteins involved in binding and entry of EBV into B-cells are listed in table 1.5. This C3d complement receptor is also known as complement receptor 2 (CR2) or CD21 and it is found abundantly at the surface of B-cells (Fingeroth *et al*, 1984; Frade *et al*, 1985).

EBV protein	**Open reading frame ***	**Known or proposed function**
gp25	BKRF-2	Complexes with gp42 and gp85
gp85	BXLF-2	Complexes with gp25 and gp42
gp42	BZLF-2	Complexes with gp25 and gp85 and binds to HLA class II
gp110	BALF-4	Virus maturation
gp350/220	BLLF-1	Initial binding by virion to CD21

Table 1.5: EBV glycoproteins involved in attachment and entry. Adapted from Speck *et al*, 2000 (Speck *et al*, 2000). HLA: Human Leucocyte Antigen. * Kieff and Rickinson, 2001.

EBV binds to CD21 on target B-cells using the gp350-gp220 complex and leading to aggregation of CD21 on the cell membrane (Tanner *et al*, 1987). This is followed by fusion of the viral envelope with the target cell membrane or by fusion with an endosomal membrane following endocytosis of EBV. Fusion is mediated by the gp25-gp42-gp85 complex binding to HLA class II loci HLA-DP, -DQ or -DR, where HLA-DP, -DQ and -DR act as co-receptors (Haan *et al*, 2000; Haan and Longnecker,

2000; Kieff and Rickinson, 2001; Li *et al*, 1997; Li *et al*, 1995). The binding of gp42 to HLA-DQ, leading to EBV entry, was restricted further to particular HLA-DQ alleles (Haan and Longnecker, 2000). The process of entry of EBV into a target B-cell is summarised in figure 1.4.

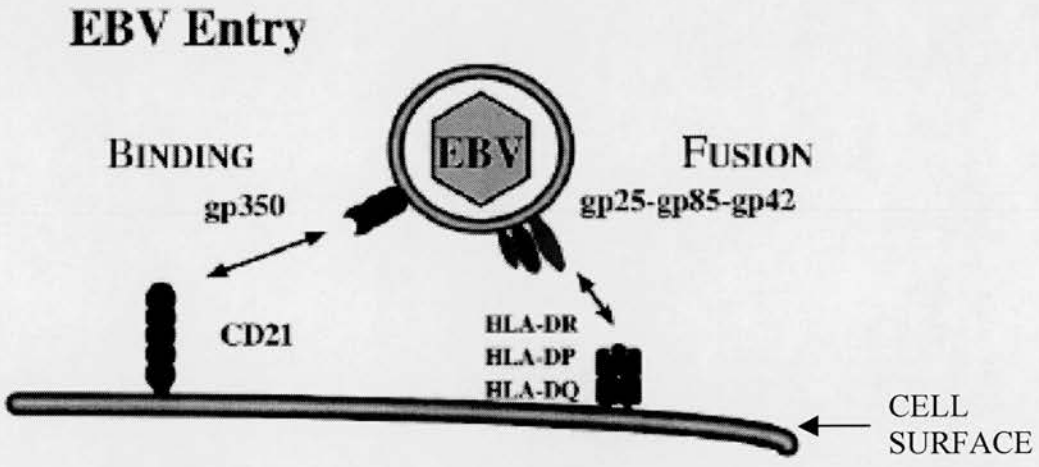


Figure 1.4: EBV entry (Speck *et al*, 2000). **Reprinted from Virology, Volume 277, Speck P, Haan KM and Longnecker R, Epstein-Barr Virus entry into cells, pages no 1-5, Copyright (2000), with permission from Elsevier.**

Whilst CD21 is the principal target cell receptor for EBV, the presence of other receptors is likely, as it has been reported that EBV deletion mutants lacking gp350/220 are able infect B-cells but also epithelial cells (Janz *et al*, 2000).

1.2.2.3 EBV infection of epithelial cells *in vivo* and *in vitro*

EBV has also been shown to infect epithelial cells of the oropharnyx *in vitro* (Sixbey *et al*, 1983) and also *in vivo* (Pegtel *et al*, 2004; Sixbey *et al*, 1984). CD21 has been detected on epithelial cells of the oropharnyx (Young *et al*, 1986). However, the mechanisms by which EBV enters epithelial cells are poorly understood, as epithelial cells do not always express CD21 and are often refractory to EBV infection *in vitro*. Research into the role of CD21 in epithelial cell infection has yielded mixed results with some researchers describing infection of CD21-negative gastric epithelial cells, implying a CD21-independent mechanism, (Yoshiyama *et al*, 1997) and others

describing the infection of 293 epithelial cells expressing low level CD21, suggesting a CD21-dependent pathway (Fingeroth *et al*, 1999). CD-21/major histocompatibility class (MHC) II-independent infection did not require gp42 or the gp220-gp350 complex but instead required the use of the gp25-gp85 complex to bind to epithelial cells (Borza and Hutt-Fletcher, 2002; Molesworth *et al*, 2000; Spear and Longnecker, 2003; Tugizov *et al*, 2003; Wang *et al*, 1998). In 2003 Tugizov *et al* proposed three CD21-independent pathways by which EBV could infect mucosal oropharyngeal epithelial cells in a human polarised tongue model and pharyngeal cells in culture. The proposed pathways are 1) by direct cell to cell contact of apical cell membranes with EBV-infected lymphocytes; 2) entry of cell-free virions through basolateral membranes, mediated in part by an interaction between β1 or α5 β1 integrins and the EBV BMRF-2 protein; and by 3) virus spread directly across lateral membranes to adjacent epithelial cells following the initial infection. BMRF-2 is an EBV envelope glycoprotein expressed in lytically infected cells (Modrow *et al*, 1992). Integrins are also involved in the binding of many other viruses to their target cells including α3β1 integrin acting as receptor for KSHV (Akula *et al*, 2002). The three CD21-independent pathways are summarised in figure 1.5.

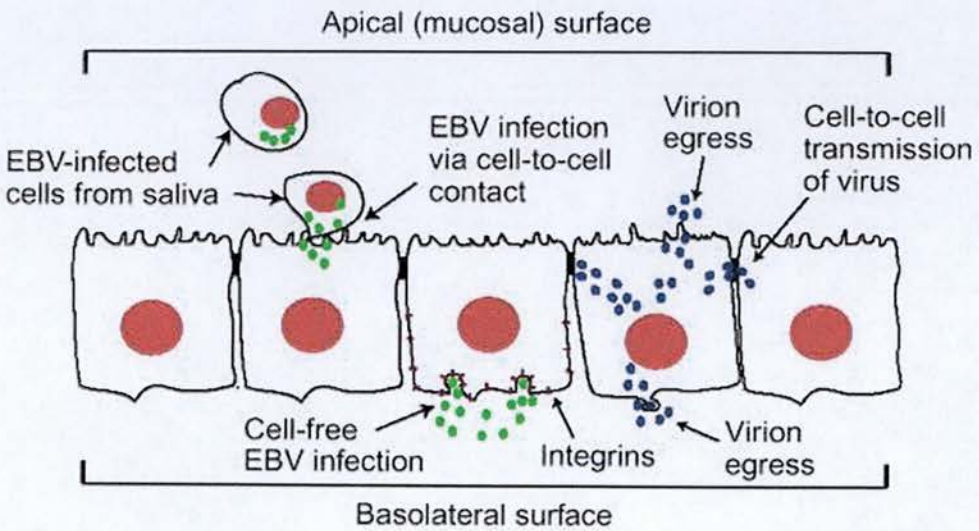


Figure 1.5: CD21-independent pathways of oropharyngeal epithelial cell infection by EBV. **Reprinted by permission from Macmillan Publishers Ltd: Tugizov SM, Berline JW and Palefsky JM. Nature Medicine, 9: 307-314, copyright (2003).**

The principal cellular receptor for EBV infection of epithelial cells has yet to be clearly identified but integrins, such as those described by Tugizov *et al* (2003), appear to be likely candidates.

Interestingly, EBV requires the gp25-gp42-gp85 complex for entry into B-cells, during which gp42 binds to MHC class II and is essential for infection (Wang and Hutt-Fletcher, 1998). However, infection of epithelial cells, which lack MHC class II, does not require gp42 but requires the gp25-gp85 complex (Borza and Hutt-Fletcher, 2002; Molesworth *et al*, 2000; Spear and Longnecker, 2003). EBV virions "produced" by B-cells lack gp42 and are thus able efficiently to infect MHC class II-negative epithelial cells but not MHC class II-positive B cells. Conversely, EBV virions "produced" by MHC class II negative-epithelial were able to infect B-cells through interactions of gp42 with MHC class II (Borza and Hutt-Fletcher, 2002). This alternate tropism may provide some clues to the natural *in vivo* infection and persistence process, which still remains an area of controversy. This is discussed in section 1.2.3.

1.2.3 EBV primary infection and persistence *in vivo*

The process of primary infection and subsequent persistence of EBV *in vivo* have been a great source of controversy and remain so to date. One hypothesis is that EBV directly infects B-cells underlying the squamous epithelium of the oropharynx. The other hypothesis is that EBV infects squamous epithelial cells of the oropharynx and subsequently the underlying B-cells.

1.2.3.1 B-cell only hypothesis

Salivary exchange was established as the transmission route for EBV by Gerber *et al* in 1972 (Gerber *et al*, 1972). Waldeyer's ring is a ring of lymphoid tissue in the throat comprising the palatine, lingual and pharyngeal tonsils. In the lymphoid tissue of Waldeyer's ring there are crypt structures that run deep into the lymphoid tissue of the tonsils (Macsween and Crawford, 2003; Perry and Whyte, 1998). Within these

crypts, epithelial cells are not so tightly bound to one another as in the remainder of the oropharyx and B-cells have been found infiltrating these crypts (Faulkner *et al*, 2000). This is a potential gateway for EBV to infect B-cells directly in primary infection, and conversely, this is likely to be a gateway for EBV to egress to the saliva for transmission to other susceptible individuals. EBV infects quiescent naïve B-cells in Waldeyer's ring and drives their differentiation to proliferating B-cell blasts in an EBNA-2-dependent fashion using the growth programme (latency III) (Babcock *et al*, 2000; Babcock and Thorley-Lawson, 2000). These B-cells are now in effect growth transformed by EBV. From that point, B-cell blasts progress to germinal centre B-cells by switching to the default programme (latency II) with the expression of LMP-1 (Babcock and Thorley-Lawson, 2000) providing a ligand-independent CD40-like activation signal to the B-cell (Gires *et al*, 1997), the expression of LMP-2A providing a ligand-independent rescue signal paralleling B-cell activation with antigen (Caldwell *et al*, 1998). Thereafter, germinal centre B-cells exit the cell cycle and become memory cells using the EBNA-1 only program (latency I) that enables episomal maintenance and replication. This drive to differentiate is the way that EBV creates its site of persistence – post-germinal centre memory B-cells (Babcock *et al*, 1998). This is summarised in figure 1.6.

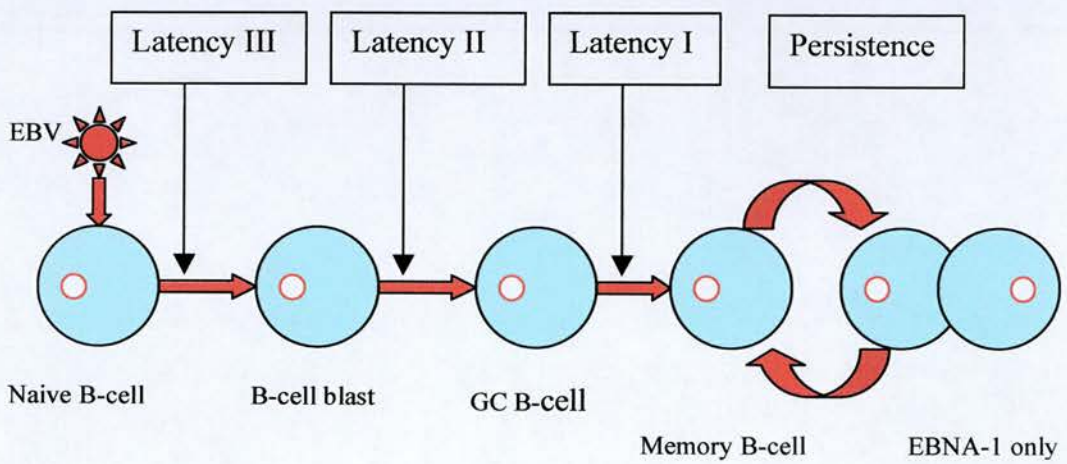


Figure 1.6: EBV infection of naïve B-cells and its transition to persistence in memory B-cells. Figure adapted from Thorley-Lawson and Gross, 2004.

Antigen activated latently infected memory B-cells can recirculate back to Waldeyer's ring, where they can differentiate into plasma cells and may switch to the lytic cycle, producing progeny virus which can be released into the saliva and infect other naïve bystander B-cells (Macsween and Crawford, 2003). Such differentiation linked with lytic replication was demonstrated *in vitro* by Crawford and Ando (1986) and *in vivo* by Laichalk & Thorley-Lawson (2005) (Crawford and Ando, 1986; Laichalk and Thorley-Lawson, 2005). Further support for this B-cell only hypothesis comes from research by Faulkner *et al* (1999), which showed that individuals with X-linked agammaglobulinaemia, who lack Bruton's thymidine kinase, and as a result have no functional B-cells, were not infected by EBV (Faulkner *et al*, 1999). These findings support the hypothesis that (naïve) B-cells are not only the site of primary EBV infection but are also the site of EBV persistence (memory) (Babcock *et al*, 1998).

1.2.3.2 Epithelial cell hypothesis

The second hypothesis is that EBV infects epithelial cells of the oropharnyx in the first instance and then the B-cells underlying the epithelium (Allday and Crawford, 1988) and that epithelial cells may also act as a site of EBV persistence. Epithelial cells were believed to be involved in EBV infection due to EBV's association with epithelial malignancies such as UNPC (discussed in section 1.3.1.1 e)) (zur Hausen *et al*, 1970) and gastric carcinoma (Shibata and Weiss, 1992). Additionally chronic low level oro-/naso-pharyngeal shedding is seen in healthy EBV carriers (Yao *et al*, 1985), which may not be arising from B-cells, as they are poor producers of virus (Allday and Crawford, 1988). Initially, epithelial cells were found to be refractory to infection *in vitro* unless EBV receptors were reconstituted within these cells (Shapiro and Volsky, 1983). But, EBV was conclusively found to infect epithelial cells *in vitro* as well as *in vivo* by Sixbey *et al* in 1983 and 1984 and its receptor, CD21, was identified on oropharyngeal epithelial cells, albeit at low level (Sixbey *et al*, 1984; Sixbey *et al*, 1983; Young *et al*, 1986). Further work supporting the epithelial cell hypothesis found that EBV originating from B-cells was "epithelial cell-tropic" and conversely, EBV originating from epithelial cell was "B-cell-tropic" (discussed in

section 1.2.2.3) (Borza and Hutt-Fletcher, 2002). This dual tropism may be a link between epithelial cells and B-cells during primary infection and it could also be the link between B-cells and epithelial cells during virus egress to the saliva. The presence of a putative link between epithelial cells and B-cells was further supported by the detection of EBV infection in *ex vivo* tonsil epithelial cell culture (Pegtel *et al*, 2004). This work suggested that epithelial cells from the tonsils of infected asymptomatic individuals were either infected *in vivo* prior to explantation or shortly post-explantation by direct contact with B-cells producing "epithelial cell-tropic" EBV in the explant, and therefore, demonstrated that epithelial cells can support EBV infection *in vivo*. Additional support has been leant to the epithelial cell hypothesis in a recent publication showing that EBV particles attached at the surface of resting B-cells, which had been recently exposed to EBV, could infect epithelial cells in cell-to-cell mediated fashion (Shannon-Lowe *et al*, 2006). This route of infection was considerably more effective than cell-free infection of epithelial cells, due to the formation of intimate virological synapses between B-cells and epithelial cells. Interestingly, the dual tropism situation detailed above could be overcome in this system, with EBV lacking gp42 binding to B-cells and being efficiently transferred to epithelial cells, suggesting that other binding mechanisms may be involved. Glycoprotein 350 was deemed essential for transfer. The transfer of EBV from B-cell to epithelial cell could be achieved within 10 minutes of cell-to-cell contact (Shannon-Lowe *et al*, 2006).

1.3 EBV: immune responses and associated diseases

1.3.1 EBV epidemiology, transmission and diseases

EBV infects in excess of 90% of the world's adult population. EBV, along with other herpes viruses, is one of the most successful pathogens at establishing a lifelong infection in its host generally without resulting in overtly pathogenic disease. This may be the result of the exceptionally long history of co-evolution between herpes viruses and man (McGeoch *et al*, 1995). EBV was first discovered in the context of BL in 1964 and since then it has been established that there are two sub-types of EBV – EBV type 1 and EBV type 2. EBV type 1 (or A) is found predominantly in the West and EBV types 1 and 2 (or B) are found equally in Africa and Papua New Guinea (Zimber *et al*, 1986). EBV was identified as the causative agent of IM in 1968 (Henle *et al*, 1968). Following primary infection, EBV establishes a persistent infection, in which the virus can remain latent in memory B-cells but can also be productive, with virus shedding in the saliva facilitating the spread of the virus to susceptible individuals. Between 5 to 500 per 10^7 circulating B-cells are EBV-infected and this level of viral load appears to remain constant over time (Miyashita *et al*, 1995). Whilst salivary exchange is the most common transmission route, sexual transmission may also occur, as EBV has been detected in genital secretions of both men and women (Israele *et al*, 1991; Sixbey *et al*, 1986). These findings were supported by research from Crawford *et al* (2002), who found that two thirds of IM cases and a tenth of asymptomatic EBV infections were associated with sexual intercourse or closely related behaviour in a cohort of 1006 university students (Crawford *et al*, 2002). The acquisition of EBV resulting in an asymptomatic infection was found to be associated with sexual behaviour in young women (aged 15 to 19 years) and increasing numbers of sexual partners increased the risk of infection (Woodman *et al*, 2005).

Since its discovery, EBV has been associated with a number of diseases, some benign and others malignant. These diseases are predominantly of lymphoid or epithelial origin but EBV-associated smooth muscle tumours have also been described. These EBV-associated diseases have been listed in table 1.6, along with cofactors/risk factors as well as the percentage of cases that are associated with EBV. There is also an inextricable association between EBV-associated diseases and immune status; therefore EBV-associated diseases will subsequently be discussed within that context. EBV-associated diseases affecting immunocompetent individuals have been highlighted in green in table 1.6 and those affecting immunocompromised individuals have been highlighted in yellow. In some cases, disease can affect both immunocompetent and immunocompromised individuals and hence these diseases have been highlighted in both green and yellow.

Disease	Cofactors/Risk factors and individuals at risk	% Association with EBV
Lymphocyte origin		
IM	Delayed primary infection	Majority of cases
BL	African children – endemic BL	97% - 100%
	Sporadic BL	10%-70%
	HIV	30% - 40%
HD	Children in developing countries	≈ 65%
	Young adults in high socioeconomic groups and/or with history of IM	≈ 80%
BLPD	PTLD	> 90%
	HIV:	
	Primary central nervous system lymphoma	<100%
	Peripheral lymphoma	≈ 50%
XLPS	♂ offspring from ♀ carriers of XLPS mutation	>90%
Primary effusion lymphoma	HIV	70% - 80% (with HHV-8)
NK/T-cell	Chronic active EBV/	10% - 100% depending on
	HIV/AIDS	histological type
Epithelial cell origin		
NPC	Southern Chinese & Inuit races	Non-keratinised 100% Keratinised 30% - 100%
Gastric carcinoma	Not identified	5% - 15% Adenocarcinoma 100% Undifferentiated carcinoma of nasopharnygeal type
OHL	HIV/immunosuppression	100%
Other cell types		
Leiomyosarcoma	HIV and other immunodeficiencies – mainly in children	Not known

Table 1.6: EBV associated diseases – in green those affecting immunocompetent individuals and in yellow those affecting immunocompromised individuals (Crawford, 2001; Hopwood and Crawford, 2000; Macsween and Crawford, 2003). Abbreviations: BLPD: B-cell lymphoproliferative disease, HIV – human immunodeficiency virus, NK cell – natural killer cell, OHL – oral hairy leucoplakia, PTLD – post-transplant lymphoproliferative disease, XLPS – X-linked lymphoproliferative syndrome.

Other diseases that have been associated with EBV include salivary gland (parotid) tumours (Raab-Traub *et al*, 1991), thymic carcinoma (Leyvraz *et al*, 1985), hepatocellular carcinoma (Sugawara *et al*, 1999) and breast cancer (Bonnet *et al*, 1999). The link with breast cancer has since been refuted but remains controversial (Perrigoue *et al*, 2005).

1.3.2 EBV diseases and immune status

In this section, some of the diseases associated with EBV, already mentioned in table 1.6, will be discussed. The presentation of diseases associated with EBV is closely linked with immune status, hence these will be discussed in two separate sections starting with diseases occurring in immunocompetent individuals, followed by those affecting immunocompromised individuals. Particular emphasis will be placed on post-transplant lymphoproliferative disease (PTLD), which will be discussed in section 1.4. PTLD occurs in up to 10% of transplant patients but it is associated with up to 60% mortality following solid organ transplantation (SOT) and up to 80% following bone marrow transplantation (BMT) (Svoboda *et al*, 2006).

1.3.2.1 Immune competence and EBV-associated diseases

a) Infectious mononucleosis

Infectious mononucleosis is a self-limiting lymphoproliferative disease that occurs in up to 25% of individuals when primary EBV infection is delayed to teenage/early adulthood and this is mostly seen in industrialised countries (Crawford *et al*, 2006). In Asia, South America and the African Sub-Continent almost all children have seroconverted in early childhood and therefore IM occurs extremely rarely (Biggar *et al*, 1978a). EBV was found to be the cause of IM in 1968 when a laboratory worker presented with acute IM symptoms and seroconverted during the course of the infection (Henle *et al*, 1968). Serology remains the main diagnostic method for IM.

The clinical presentation of IM can include fever, fatigue, pharyngitis, lymphadenophathy, hepatosplenomegaly and in rare cases it can also include rashes

such as urticaria (Macsween and Crawford, 2003). Infectious mononucleosis' symptoms usually resolve within two to four weeks, although in extremely rare cases the symptoms can persist constantly or intermittently as chronic active IM (Crawford, 2001). The speed of resolution of symptoms may be associated with the number EBV antigen epitopes (latent and lytic) targeted by CD8+ T-cells specific for EBV antigens, as it was noted in a study that one patient (patient 1) with CD8+ T-cells reactive to seven epitopes had a much shorter course of acute symptoms (2-3 weeks) in comparison to a patient (patient 2) with CD8+ T-cells reactive to only two epitopes who experienced acute symptoms for four months (Bharadwaj *et al*, 2001). Interestingly, the CD8+ T-cells from patient 1 were less cytotoxic in an *ex vivo* assay than those of patient 2, so it was suggested that a broader but less cytotoxic response may be more beneficial (Bharadwaj *et al*, 2001). Furthermore PBMC-associated EBV DNA viral load remained detectable in patient 2 five months post-diagnosis whilst patient 1 had no detectable EBV DNA following the acute phase of IM (Bharadwaj *et al*, 2001). In another study covering the course of the convalescence phase (6 months following diagnosis), EBV DNA loads associated with PBMCs and in plasma were found to decrease overall, with some rebound peaks in EBV DNA loads (Fafi-Kremer *et al*, 2005). This decrease EBV DNA load was not seen in the saliva where EBV DNA loads remained elevated (Fafi-Kremer *et al*, 2005). So, whilst patients may become asymptomatic following the acute phase of the disease they can remain infectious throughout the convalescence phase.

The process of EBV primary infection is described in section 1.2.3 and the immunological response, which induces the symptoms of IM, in particular the cellular immune response, is discussed in section 1.3.3.2. The humoral response is discussed in section 1.3.3.1.

b) Burkitt's lymphoma

BL was first identified in African children by Dennis Burkitt in 1958, and it remains the most common childhood malignancy in Central Africa. It usually presents as a tumour of the jaw but it may also present at other anatomical sites such as the gastrointestinal tract, the kidneys and the liver. There are two types of BL – endemic BL, that occurs in equatorial Africa and Papua New Guinea and sporadic BL that occurs worldwide though the frequency of sporadic BL is 50-fold less than endemic BL (Rickinson and Kieff, 2001; Young and Murray, 2003). There are three key factors in the pathogenesis of BL – EBV, the dysregulation of the oncogene *c-myc* and malaria. EBV is detected in approximately 20% (ranges from 10% to 70%) of sporadic BL tumours whereas it is detected in over 97% endemic BL tumours (Macsween and Crawford, 2003; Rickinson and Kieff, 2001). BL tumours comprise rapidly proliferating small B-cells (which are "trapped" in the cell cycle and morphologically resemble germinal centre B-cells), and occasional infiltrating macrophages (Crawford, 2001; Rickinson and Kieff, 2001; Thorley-Lawson and Gross, 2004). BL cells exhibit a latency I expression profile, in which EBNA-1 maintains the virus in the cell and EBERs possibly contribute to pathogenesis by inducing IL-10 production and upregulating the anti-apoptotic factor *bcl-2* (Kitagawa *et al*, 2000; Komano *et al*, 1999). BL cells consistently display one of three chromosomal translocations (8:2, 8:14 and 8:22) where the *c-myc* oncogene, found on chromosome 8, comes under the transcriptional control of the immunoglobulin (Ig) heavy chain locus on chromosome 14 or the Ig light chain loci (lambda on chromosome 22 and kappa on chromosome 2) and becomes constitutively expressed (Crawford, 2001; Leder *et al*, 1983). These translocations are observed regardless of EBV association. Malaria is strongly linked with endemic BL, as tumours occur only in Papua New Guinea and equatorial Africa where malaria is holoendemic (Rickinson and Kieff, 2001). Furthermore endemic BL levels diminished in areas of Papua New Guinea where mosquito eradication programmes were brought in (Crawford, 2001), thus corroborating the link between endemic BL and malaria. Acute malaria induces a period of T-cell immunosuppression (Whittle *et al*, 1984) and children suffering from acute malaria were found to have up to five times the

number of EBV-infected cells in peripheral blood than in convalescent malaria, suggesting a lack of cytotoxic T-cell control of EBV-infected cells (Lam *et al*, 1991). It has been postulated that this increase in EBV-infected cells may be associated with an increased risk of genetic cellular abnormalities due to the high cell turnover which may in turn contribute to the evolution/development of BL (Whittle *et al*, 1984).

c) Hodgkin's disease

HD is the most common lymphoma in young individuals in the Western world. HD occurs worldwide, with two age peaks of disease onset – one in childhood and the other in the over 50's but there are socio-economical/ geographical variations in the time of disease onset (Macsween and Crawford, 2003). In more affluent societies, the first peak of HD occurs between the ages of 15 and 35 and in less affluent societies this occurs between the ages of 5 and 10. This delay in disease presentation in the West parallels what has been seen in EBV infection and IM and pointed to a link between HD and EBV (MacMahon, 1966). This link was undeniably established by the detection of EBV genomes in tumour biopsies and specifically in the malignant mononuclear Hodgkin cells and multinuclear Reed-Sternberg cells (HRS cells) (Weiss *et al*, 1989). HD comprises four subtypes – nodular sclerosing (NS), mixed cellularity (MC), lymphocyte depleted (LD) and lymphocyte predominant (LP) (Rickinson and Kieff, 2001). NS, MC and LD HD are collectively known as "classic HD" in which HRS cells are of monoclonal B-cell origin based on their immunoglobulin gene (*Ig* gene) rearrangement. These HRS cells are of germinal centre origin (Thorley-Lawson and Gross, 2004), and some bear "crippling" *Ig* gene mutations following somatic hypermutation that are not generally consistent with cell survival *in vivo* (Mancao *et al*, 2005). HD HRS cells exhibit latency type II EBV antigen expression pattern, therefore expressing the viral antigens (LMP-1 and LMP-2A), which promote the survival of these otherwise non-viable cells. Worldwide, between 60% to 80% of MC and LD tumours and 20% to 40% of NS tumours are EBV positive and less than 10% of LP tumours are EBV positive (Rickinson and Kieff, 2001); however, these percentages are subject to geographical variations. HD

is another example of EBV's versatility in causing disease by differential expression of the necessary antigens for the survival of abnormal B-cells.

d) T-cell lymphoma

EBV was first detected in T-cells in 1988 in patients with chronic active EBV infections (Jones *et al*, 1988; Kikuta *et al*, 1988). EBV entry into T-cells can occur via CD21, which has been detected at the surface of activated T-cells (Fischer *et al*, 1991), however CD21 is not detected consistently on all tumour T-cells (Jones *et al*, 1988; Kikuta *et al*, 1988). T-cell lymphomas can be separated into two main categories: virus-associated haemophagocytic syndrome (VAHS) T-cell lymphomas and nasal NK/T-cell lymphomas (Rickinson and Kieff, 2001). VAHS in children is intrinsically linked with EBV: T-cell proliferation is driven by EBV infection leading to haemophagocytosis (Rickinson and Kieff, 2001). Nasal NK/T-cell lymphomas mainly affect adults of Asian, Latin and Native American origin (Nava and Jaffe, 2005). These lymphomas primarily result in the destruction of the bone structure of the nasal cavity, although other anatomical sites can be involved (skin, gastrointestinal tract, testis and upper respiratory tract) which is a poor prognostic factor (Nava and Jaffe, 2005). Both of these lymphoma types express EBV antigen in a latency I/II pattern, as LMP-1 expression is low to almost undetectable. All of these lymphomas are associated with EBV (Rickinson and Kieff, 2001). The prognosis of both VAHS and NK/T-cell lymphomas is poor.

e) Nasopharyngeal carcinoma

NPC is a tumour of the squamous epithelium of the nasopharynx. NPCs can be broadly separated into two categories based on the differentiation of the epithelial tumour cells: differentiated (keratinised) and undifferentiated/poorly differentiated (non-keratinised) (Rickinson and Kieff, 2001). NPC occurs worldwide at a relatively low incidence of 0.5 per 100000 but this increases to 20 to 30 per 100000 in southern China and the Inuit population (Crawford, 2001; Murray and Young, 2001; Rickinson and Kieff, 2001). Interestingly, individuals of Chinese descent have a higher incidence of the disease regardless of geographical location, suggesting a

genetic susceptibility, which may be linked to HLA type (Lu *et al*, 1990; Young and Rickinson, 2004). Other contributory factors to the disease include a diet rich in salted/preserved foods such as fish that contains carcinogenic nitrosamines, as well as the consumption of beer and strong alcohols and fresh pork/beef offal (Armstrong *et al*, 1998). The use of Chinese herbal snuff is also associated with NPC (Yu and Yuan, 2002).

The association between EBV and NPC was first established using serological methods, in which antibodies generated to antigens in BL cells lines reacted with serum from UNPC/NPC patients (Old *et al*, 1966). This link was further substantiated when EBV DNA was detected in tumour biopsies by *in situ* hybridisation (Wolf *et al*, 1973), and then, using Southern blotting, it was also shown that EBV infection occurred prior to clonal expansion of malignant cells (Raab-Traub and Flynn, 1986). There is up to a 100% association between undifferentiated/poorly differentiated forms of NPC and EBV, however this association may decrease in more keratinised forms of the disease (Rickinson and Kieff, 2001). Nasopharyngeal tumours express the latency II EBV gene expression programme (see table 1.3). EBERs, BARTs, LMP-2A and -2B transcripts are readily detectable but the LMP-1 protein is not always detectable, being absent in approximately 65% of tumours (Rickinson and Kieff, 2001). The EBNA-1 protein is the only EBNA to be readily detectable in NPC (Lee, 2002). Whilst, high IgG and IgA antibody levels to the lytic VCA and EA act as a marker for NPC and increase with NPC severity (Henle and Henle, 1976), no lytic form of the genome has been detected in cells from NPC tumour biopsies.

1.3.2.2 Immune deficiency and EBV-associated diseases

Immune deficiency may be of an acquired, inherited or iatrogenic nature. In each of these situations EBV can give rise to diseases such as acquired immunodeficiency syndrome (AIDS)-related lymphomas and oral hairy leucoplakia (Opelz and Dohler, 2004), in the case of acquired immune deficiency; X-linked lymphoproliferative syndrome (XLPS) and B-cell lymphoproliferative disease (BLPD), in the case of

inherited deficiency and PTLD, in the case of iatrogenic immune deficiency. PTLD will not be discussed in this section but will be discussed at length in section 1.4.

a) Acquired immunodeficiency syndrome-related lymphomas

HIV-infected individuals have a high risk of lymphoma development due to the T-cell immunosuppressive nature of the disease. The diseases that occur in HIV/AIDS patients are listed in table 1.6. Sporadic BL can occur in HIV infected individuals (Ziegler *et al*, 1982) where it is an AIDS-defining illness (Crawford, 2001). In such cases there is a 30% to 40% association with EBV. BLPD, which encompasses primary central nervous system (CNS) lymphoma and peripheral lymphoma, occurs in end-stage AIDS. EBV is associated with nearly all primary CNS lymphomas and approximately 30% to 50% of peripheral lymphomas (MacMahon *et al*, 1991). Primary effusion lymphoma (PEL) also affects AIDS patients, though it is rare. PEL is EBV positive in 70% to 80% of cases (HHV-8 is also associated with PEL). Finally, in rare cases, HD can arise in HIV-positive individuals where there is a 100% association with EBV (defined by LMP-1 detection) (Audouin *et al*, 1992).

b) Oral hairy leucoplakia

OHL is a benign condition affecting the epithelium of the tongue, particularly the edges of the tongue, which is caused by EBV lytic replication and presents predominantly in AIDS patients (Greenspan *et al*, 1985). However OHL can affect other immunosuppressed individuals, such as renal transplant recipients (Itin *et al*, 1988). The association with lytic replication of EBV was corroborated when acyclovir treatment coincided with complete resolution of OHL lesions, in which only EBV could be detected (Resnick *et al*, 1988). The replication of EBV associated with OHL, as well as EBV DNA detection were restricted to the upper epithelium of the tongue (Thomas *et al*, 1991).

c) X-linked lymphoproliferative syndrome

XLPS or Duncan's Disease was identified over 30 years ago when a child from the Duncan family, in which five other maternally-related males had died, succumbed to IM and was found to have a thymus gland, lymph nodes and spleen depleted of T-cells (Purtilo, 1983). In XLPS a primary EBV infection can present as fulminant IM with acute B-cell proliferation, which is fatal in over 50% of cases. This occurs because individuals with XLPS cannot control a primary EBV infection and those who survive the acute phase of the disease may go on to develop lymphoma and hypogammaglobulinaemia (Williams and Crawford, 2006). The product of the XLP gene is SAP (signalling lymphocytic-activation molecule (SLAM) associated protein) which is found in the cytoplasm of activated T-cells and NK cells (Coffey *et al*, 1998). SAP interacts with the cytoplasmic domain of SLAM or CD150, which is found at the surface of B- and T-cells (Sayos *et al*, 1998). The SAP acts as a cell activation modulator by binding to SLAM, which is a member of the immunoglobulin superfamily, as well as to four other member of the immunoglobulin superfamily, including 2B4 or CD244 found on NK cells and cytotoxic T-cells (Nakajima *et al*, 2000; Sayos *et al*, 1998). Based on this, it has been postulated that the interaction between SAP and SLAM acts as a control in the balance of the production of Th1/Th2 cytokines in response to cellular activation (Williams and Crawford, 2006). Mutations affecting SAP may therefore compromise this balance resulting in a lack of cytotoxic T-cell activation by helper T-cells because these could not respond to antigen presentation by EBV-infected B-cells (Sayos *et al*, 1998). Additionally, SAP mutations disrupt humoral responses in a cytokine-independent fashion by affecting the expression of extracellular molecules essential in T-cell-B-cell collaboration necessary in the generation of an appropriate humoral response (Cannons *et al*, 2006). It has also been shown that SAP mutations could detrimentally affect NK cell cytotoxicity towards EBV infected cells. This is because mutated SAP failed to act on downstream activation pathways following the binding of 2B4 on NK cells with CD48 expressed at the surface of EBV-infected cells (Nakajima *et al*, 2000). Additionally the interaction of 2B4 with CD48 in XLPS individuals resulted in further NK cell cytotoxicity inhibition (Parolini *et al*, 2000).

Most individuals with XLPS do not survive beyond their teenage years and treatment of XLPS is limited, though the monoclonal antibody to CD20 (expressed by B-cells), also known as rituximab, has been used successfully in two patients who remain disease free two years post-primary infection (Milone *et al*, 2005).

1.3.3 The immune response to EBV

As over 90% of the adult population is infected by EBV, but most remain asymptomatic, the immune response to the virus plays a critical role. The importance of the immune control is highlighted by immunocompromised individuals who often present with severe EBV-associated diseases. Before describing the EBV-specific immune response, the general immune response to viruses will be reviewed.

1.3.3.1 General immune response to viruses

Viruses are obligate intracellular pathogens. Innate immunity to viruses is centred on the production of type I interferons (IFN), which include IFN-α and -β. The secretion of type I IFN is induced by two broad types of receptors: toll-like receptors and cytosolic receptors. Both receptor types detect viral nucleic acids. The cytosolic receptors are ubiquitous and found in all cell types, whereas the toll-like receptors operate in endosomes of specialised cells (dendritic cells and macrophages) (Stetson and Medzhitov, 2006). The stimulation of these receptors induces the production of type I IFN, which acts in an autocrine and paracrine fashion, inducing an antiviral state. This antiviral state is mediated by antiviral proteins (enzymes), which respond to double-stranded RNA. One family of antiviral proteins is the 2'5' ogliadenylate synthases family, which produce 2'5'oligoadenylates that stimulate RNases to degrade both host and viral RNA. P1 kinase, another antiviral protein, is also activated by double stranded RNA and inhibits translation of both viral and host messenger RNA. Both of these antiviral responses seek to halt viral replication and lead to apoptosis of the cell, which prevents the spread of the virus to neighbouring cells. In uninfected cells, type I IFNs induce the expression of MHC class I making them resistant to NK cell activity, as downregulation of MHC class I can be induced

by viral infection. This makes newly infected cells more susceptible to CD8+ cytotoxic T-cells and NK cell lysis, due to increased antigen presentation via MHC class I leading to more efficient elimination of the virus.

Apopotic cells and debris from lysis of virus-infected cells are subsequently taken up by macrophages and dendritic cells or tissue-derived dendritic cells (if the infection occurs in a non-lymphoid organ). This will lead to antigen presentation to CD4+ and CD8+ T-cells in lymph nodes. Where the CD4+ T-cells and CD8+ T-cells recognise antigen in an MHC class II and class I manner respectively, the cells undergo clonal expansion. Appropriate activation of both cell types requires not only the interaction of the T-cell receptor (TCR) with the MHC/peptide complex but also the interaction of CD28, which is a receptor involved in the regulation of T-cell response, by providing a co-stimulatory signal mediated by its ligands CD80 and CD86 (CD80 and CD86 were known as B7.1 and B7.2 respectively) (Lenschow *et al*, 1996). CD27, another cell surface marker, interacts with CD70 (which is expressed by EBV-infected lymphoblastoid cells) (Yamada *et al*, 2002). Both CD27 and CD28 expression decreases following antigenic stimulation and they are not re-expressed (Appay *et al*, 2002a). Additionally, CD45RA (naïve T-cell marker) and CD45RO (antigen-experienced T-cell marker) expression change following antigen exposure with a decrease in CD45RA and an increase in CD45RO expression. Many other markers are upregulated or downregulated following antigen encounter, although CD27 and CD28, in conjunction with CD45RA expression, have been repeatedly used in studies to establish the differentiation status of CD4+ T-cell and CD8+ T-cells in response to different viral infections (including EBV) (Appay *et al*, 2002a; Appay *et al*, 2002b).

The generation of effective immune memory in terms of virus-specific CD8+ T-cells relies on the presence of CD4+ T-cells during the course of the primary immune response. In the absence of CD4+ T-cells, the primary CD8+ T-cell response takes place but upon antigen re-exposure these CD8+ T-cells do not respond (Bevan, 2004).

The humoral immune response also develops with the help of CD4+ T-cells providing essential B-cell specific cytokines (such as interleukin-4 and -5). Virus-specific antibodies can be detected in the late phase of an infection. These may prevent cell-to-cell infection and may also confer resistance to further infection. Additionally antibodies may neutralise virus particles released from infected cells thus preventing spread within the body and to susceptible individuals (Guidotti and Chisari, 2001).

As the majority of individuals infected with EBV undergo silent seroconversion, information on the primary immune response to EBV is based on studies of IM patients.

1.3.3.2 The humoral immune response to EBV

Primary EBV infection has been shown to occur at an earlier age in lower socioeconomic groups than in higher socioeconomic groups (Biggar *et al*, 1978b). It is usually asymptomatic but where there is a delay in primary infection to adolescence/early adulthood this can result in IM in 25% of cases (Crawford *et al*, 2006). In IM patients EBV elicits both a humoral and a cellular response. The primary humoral response can be demonstrated by enzyme-linked immunosorbent assay and immunofluorescence detection of IgM and IgG antibodies to viral capsid antigen (VCA) and early antigen (EA), of IgM and IgG antibodies to the membrane antigen (MA, comprising gp350/220 and gp85) and the absence of IgG antibodies to EBNA (Moss *et al*, 2001). In late IM, the pattern of detectable antibodies changes with the detection of IgG antibodies only to EBNA, VCA and MA but no longer to EA (Moss *et al*, 2001). The detection of these antibodies still forms part of IM diagnosis today, along with the detection of heterophile antibodies using the Paul-Bunnel and Monospot tests. Heterophile antibodies are IgM antibodies which react with antigens of unrelated species, such as sheep and horse (agglutination of sheep red blood cells forms the basis of the Paul-Bunnel test and agglutination of horse red blood cells forms the basis of the monospot test). Heterophile antibodies can be detected in approximately 85% of IM cases. All of the IgG antibodies generated

above, but in particular the neutralising antibodies to gp350/220 (part of MA) (Thorley-Lawson and Geilinger, 1980; Thorley-Lawson and Poodry, 1982), along with IgA antibodies against gp350/220 found in the serum of 21% to 30% and in the saliva of 12% to 19% of infected individuals, may serve to prevent superinfection with other EBV isolates (in EBV seropositive individuals) and further spread of the virus to susceptible individuals (Yao *et al*, 1991).

1.3.3.3 The cellular immune response to EBV

a) NK cell response

NK cells form an integral part of the innate immune response to viral infection, including EBV. In one study, higher NK cell levels in IM patients corresponded with a lower viral load (Williams *et al*, 2005) and the authors suggested that NK cell activity may have an impact on the outcome of primary EBV infection.

b) CD8+ T-cell response

The cellular immune response to a primary EBV infection as detected in the peripheral circulation is characterised by an antigen driven "atypical" lymphocytosis comprising phenotypically activated CD8+ T-cells, which were found to be monoclonal or oligoclonal based on their T-cell receptor (TCR) usage within expanded families of the variable β-chain (Vβ) (Callan *et al*, 1996). These "atypical" CD8+ T-cells were frequently found to recognise lytic antigen epitopes, from the immediate early antigens BRLF-1 and BZLF-1 but also from other early lytic antigens such as BMRF-1, BMLF-1 and BALF-2 (Steven *et al*, 1997). The number of CD8+ T-cells specific for latent antigen was comparatively low during the course of the primary response, with only up to 2.2% of CD8+ T-cells being specific for an EBNA-3A latent epitope in the HLA-B8 context compared to 44% of CD8+ T-cells being reactive to the lytic BZLF-1 peptide (Callan *et al*, 1998). The number of CD8+ T-cells reactive to lytic antigens generated during the acute phase of the primary infection decreased dramatically and in some cases became completely undetectable as the infection progressed (Hislop *et al*, 2002), with the most expanded CD8+ T-

cells clones in the primary response being the most heavily culled (Callan *et al*, 2000). The CD8+ T-cells evolve in parallel to the progression from primary infection with lytic replication to persistent phase with latent replication. Thus, CD8+ T-cells specific to latent antigens are detected in long-term carriers (following resolution of the primary infection) and must be involved in immunosurveillance. These CD8+ T-cells are reactive to latent antigen and in particular EBNA-3 antigens (Murray *et al*, 1990; Rickinson and Moss, 1997). The immunodominant peptides to which CD8+ T-cells respond have been mapped for specific HLA types (Moss *et al*, 2001). These immunodominant peptides included EBNA-1 peptides to which CD8+ T-cells respond strongly (when EBNA-1 presentation inhibition by the glycine-alanine repeat was overcome).

Phenotypically, EBV-specific CD8+ T-cells progressed from a naïve phenotype (CD27+ CD28+ CD45RA+) to an antigen-experienced phenotype (CD27+/- CD28+ CD45RA+/-) following the model proposed by Appay *et al* (2002a). This phenotypic change was associated with acquisition of the cytolytic proteins perforin and granzyme A. Further differentiation can take place, leading to the loss of CD27 and CD28 expression and increasing levels of perforin. The antigen-experienced phenotype was associated with cells with a greater potential to proliferate whereas those having undergone further differentiation had greater cytotoxic potential but a reduced ability to proliferate.

c) CD4+ T-cells

The analysis of CD8+ T-cell responses to EBV was greatly facilitated by using MHC class I EBV antigen tetramers (Callan *et al*, 1998) but this was not feasible for CD4+ T-cells as MHC class II EBV antigen tetramers were/are not available. However, CD4+ T-cells from healthy blood donors have been shown to respond consistently to EBNA-1 presented by dendritic cells (DC) and LCL *in vitro* (Münz *et al*, 2000). Further work by the same group found that these EBNA-1-reactive CD4+ T-cells exhibited T-helper (Th) type 1 properties by secreting IFN-γ and inducing EBNA-1-specific lysis of target cells (Bickham *et al*, 2001). EBNA-1 was subsequently found

to be the most immunogenic EBV latent antigen (compared with EBNA-3C, LMP-1 and LMP-2 respectively) for CD4+ Th1 T-cell from healthy EBV carriers (Leen *et al*, 2001). This hierarchy of immunogenicity is unlike that seen in CD8+ T-cells in which the EBNA-3 family induces the strongest response, followed by EBNA-1, LMP-2, EBNA-2, EBNA-LP and LMP-1 (Leen *et al*, 2001). Moreover, CD4+ T-cells from healthy EBV carriers were shown to have an effector role, as their addition prevented the proliferation of newly EBV infected CD23+ B-cells in culture and conversely the removal of these CD4+ T-cell allowed newly EBV infected CD23+ B-cells to grow in culture (Nikiforow *et al*, 2001). This effector role was ascribed to IFN-γ secretion and Fas/FasL interaction between CD4+ T-cells and target B-cells and not to perforin secretion (Nikiforow *et al*, 2003). However, perforin was detected in CD4+ T-cells of healthy blood donor but also in the CD4+ T-cells of individuals undergoing a primary EBV infection in whom cytotoxic activity was mediated by perforin (Appay *et al*, 2002b). Another group of researchers generated EBV-specific CD4+ cytotoxic T-cells *in vitro* and found that these cells did not express perforin but instead expressed granulysin as a potential effector of cytotoxicity (Sun *et al*, 2002). Whilst these findings do not clearly establish how CD4+ T-cells respond to EBV latent antigen they do show that CD4+ T-cells have the ability to respond to antigen beyond their helper functions.

Studies into CD4+ T-cell responses to EBV latent and lytic antigens in primary EBV infection (IM) were only recently undertaken by Amyes *et al* (2003) and Percopio *et al* (2003). The mean percentage of CD4+ T-cells from IM patients responding to EBV lysate (the lysate was shown to contain BZLF-1, BMRF-1 and LMP-1) was 1.4% (ranging from 0.04% to 5.2%) (Amyes *et al*, 2003). EBV-specific CD4+ T-cells were detected in all IM patients studied, with BZLF-1-specific CD4+ T-cell detectable in 100%, BMLF-1 and EBNA-3A CD4+ T-cells detectable in 78%, EBNA-1-specific CD4+ T-cells in 33% of IM patients (Precopio *et al*, 2003). Most patients were found to respond to two or more proteins. The frequencies of CD4+ T-cells specific for these antigens in the IM patients were 1.75% for BZLF-1 and <1% for BMLF-1, EBNA-1 and EBNA-3A. The frequencies of all antigen-specific CD4+

T-cells dropped below 0.12% six weeks post-diagnosis. At one year post-diagnosis almost all EBV antigen-specific CD4+ T-cells were undetectable in all IM patients (Precopio *et al*, 2003), however, other investigators have detected CD4+ T-cells specific for latent EBV antigens in healthy EBV carriers (see paragraph above). So, whilst CD4+ T-cells do expand following a primary EBV infection, they comprise only a small fraction of the lymphocytosis seen in IM.

In contrast to CD8+ T-cells, CD4+ T-cells progressed from a naïve phenotype (CD27+ CD28+ CD45RA+ CD45RO- perforin-) to antigen-experienced phenotype (with a greater ability to proliferate and promote B-cell differentiation) without the loss of CD27 and CD28 and without the acquisition of perforin or granzyme A (CD27+ CD28+ CD45RA+/- CD45RO+) (Appay *et al*, 2002b). The acquisition of these cytolytic proteins was concomitant with the loss of CD27 and CD28 (CD45RA+/- CD45RO+) indicating that the CD4+ T-cells had undergone further differentiation.

1.4 Post-transplant lymphoproliferative disease

1.4.1 A brief history of transplantation

Transplantation of human teeth took place in ancient Egypt, Greece, Rome as well as in pre-Columbian North and South America. Since then the discipline of transplantation has thankfully evolved, although not without considerable ups and downs. The successful autografting of a kidney in a dog in 1908 led to much developmental work being undertaken in both animals and humans, without success in most cases. This *status quo* of failures remained until the immunosuppressive agent 6-mercaptopurine was discovered. In 1962 a modified, less toxic and more effective version of 6-mercaptopurine, azathioprine, was used in a kidney transplant leading to an uncomplicated post-operative course. The next key step in transplantation was the pioneering use of the fungal metabolite CsA in 1978. It was first used in seven renal transplant patients with encouraging post-transplant outcomes with five patients supporting functioning allografts (Calne *et al*, 1978). This prompted its extended use in 32 kidney, two liver and two pancreatic transplants, in which no kidney was lost through rejection (Calne *et al*, 1979) and CsA has been in use ever since. In the meantime, the first lung transplant and the first heart transplant were carried out in 1963 and 1967 respectively and in 1970 the HLA complex was discovered, which is of paramount importance in transplantation. At present, as well as heart, kidney, lung and liver transplants, pancreas, corneal, skin and small bowel transplants are also carried out (Hakim, 1997) and on the 30th November 2005 the first face transplant was carried out successfully by French surgeons in Amiens. Whilst a number of surgical/technical hurdles have been overcome in transplantation, there are post-transplantation complications, such as PTLD, which remain serious. Beyond post-transplantation complications, the lack of organ donors is probably the main limitation in transplantation compared to previous technical limitations.

1.4.2 PTLD

1.4.2.1 Incidence of PTLD

The incidence of PTLD in haematopoietic stem cell transplants is approximately 1% and in renal, heart and liver transplants this ranges from 1% to 3%, although figures of up to 10% have also been published when lung transplant patients were included (Burns and Crawford, 2004; Gottschalk *et al*, 2005). An incidence of PTLD of 1% is approximately 20 times the incidence of lymphoma in the general population (Newstead, 2005). The incidence of PTLD in intestinal transplants has been reported to be approximately 20%, although an incidence reaching up to 26.8% in paediatric recipients (9.3% in adults) has been observed (Reyes *et al*, 1996) and this is believed to be due to the large amount of lymphoid tissue associated with an intestinal transplant (Gottschalk *et al*, 2005; Newstead, 2005). PTLD is most likely to occur in the year following the transplant due to the prolonged and intense immunosuppression required to prevent graft rejection (Opelz and Henderson, 1993). The incidence of PTLD one year post-transplant represents one fifth of the cumulative incidence over 10 years with a median PTLD occurrence time of 5 years post-transplant (Opelz and Dohler, 2004). The incidence of PTLD in solid organ transplant (SOT) is dependent on a number of risk factors and these are discussed below.

1.4.2.2 Risk factors

a) T-cell immunosuppression

Penn *et al* recognised an increase in the incidence of lymphomas in transplant patients in 1969 (Penn *et al*, 1969). In 1979 following the introduction of CsA a dramatic rise in the number of cases of PTLD was noted and this was reduced substantially following a decrease in the dose of CsA used (Beveridge *et al*, 1984; Starzl *et al*, 1984). In 1980, Crawford *et al* were the first to identify an EBV-associated lymphoma in a kidney transplant patient on CsA imuunosuppressive treatment, thus establishing the link between immunosuppression and EBV-positive

lymphoma development, as such lymphomas are not seen in immunocompetent individuals. T-cell immunosuppression is a major risk factor in the development of PTLD. Other immunosuppressive agents are now in use such as tacrolimus, sirolimus and prednisolone alongside antiproliferative immunosuppressants such as azathioprine and mycophenolate mofetil (Newstead, 2005). Monoclonal antibodies such as OKT3 that bind CD3, and basiliximab and dacluzimab that bind the CD25 receptor, all of which deplete circulating T-cells, are also in use. Basiliximab and dacluzimab are used prophylactically to prevent graft rejection (BNF, 2006; NICE, 2006), whereas OKT3 has been used for both the treatment and prevention of graft rejection. The use of OKT-3 alone (Swinnen *et al*, 1990), tacrolimus alone (Cox *et al*, 1995) or a combination of both (with high tacrolimus doses) (Sokal *et al*, 1997) has been associated with a increased risk of PTLD, but compared to CsA, tacrolimus reduces the incidence of graft rejection by 11% (Hakim, 1997), highlighting a fine balance between PTLD development and graft rejection. However, a retrospective study covering 30 years of heart and lung transplants in one centre found there to be no correlation between immunosuppressive regimens consisting of OKT3, azathioprine, prednisolone, mycophenolate mofetil and tacrolimus and PTLD development, with the exception of high-dose CsA (Gao *et al*, 2003). Prolonged and/or intense periods of immunosuppression have both been associated with an increased risk of developing PTLD. Such periods would be seen in individuals with heart, lung and liver transplants or individuals who are undergoing a second transplant in whom the incidence of PTLD is five times greater than in other transplant patients (Swerdlow *et al*, 2000).

b) EBV seronegativity

EBV seronegativity prior to transplantation is another a critical risk factor, as EBV can be acquired naturally but it can also be transmitted through the transplanted organ and lead to a primary infection and seroconversion (Haque *et al*, 1996). EBV seronegative recipients of an EBV positive graft have between a 10-fold and 75-fold increased risk developing PTLD as compared with EBV seropositive recipients (Cockfield, 2001). This increased risk arises because seronegative individuals have

no immunity to EBV nor do they have the ability to mount a response due to iatrogenic immunosuppression. This is of particular relevance in paediatric transplant recipients who are highly likely to be seronegative.

1.4.2.3 Tumour classification

The term PTLD disease refers to a heterogeneous group of tumours that are principally of B-cell origin, although T- and NK-cell PTLD tumours have also been observed (Nalesnik, 2001). Tumour cells generally express latency III pattern, although some cells within a tumour may express latency I and II pattern also (Cen *et al*, 1993). Antigen expression is therefore heterogeneous. PTLD tumours are distinct from other EBV-associated tumours and they are histologically classified as 1) hyperplastic PTLD (early lesions), 2) polymorphic PTLD and 3) lymphomatous PTLD (monomorphic PTLD) (Nalesnik, 2001). Progression from early lesions to monomorphic PTLD has been documented (Larratt *et al*, 2001). Monomorphic lesions are the most aggressive and the most likely to be resistant to treatment (treatment is discussed in section 1.4.3) (Williams and Crawford, 2006). In some cases these lesions also carry genetic mutations in the *p53* tumour suppressor gene, the *ras* and/or *c-myc* oncogenes, which may contribute to disease development (Knowles *et al*, 1995). PTLD tumours can be monoclonal, oligoclonal or polyclonal with regards to their *Ig* gene rearrangement or cell surface/cytoplasmic Ig expression and tumours arising in the same patient may exhibit variation in their clonality (Cleary and Sklar, 1984; Hanto *et al*, 1982; Shearer *et al*, 1985).

1.4.2.4 Pathogenesis

EBV DNA was detected in tumour B-cells in PTLD patients (Young *et al*, 1989). The cellular origins of PTLD are varied and not limited to memory B-cells. This was established by genotyping of the immunoglobulin heavy chain (IgH) of the B-cell receptor (BCR). The PTLD tumour cells consisted of either antigen-experienced cells that had undergone BCR somatic hypermutation or naïve B-cells that had not (Timms *et al*, 2003). It was also established that B-cells bearing random BCR mutations, as well as mutations leading to a sterile genotype, which would apoptose

in immunocompetent individuals, were present in PTLD tumours (Timms *et al*, 2003). B-cell receptor-deficient cells have also been found in PTLD tumours and are prone to apoptose rapidly in a mouse model (Lam *et al*, 1997). As these tumour cells are EBV-infected, it appears highly likely that EBV provided the cells with the necessary signals for survival. LMP-1 would act as a CD40 homologue, therefore providing proliferation and activation stimuli, and LMP-2A as a BCR homologue (Bechtel *et al*, 2005; Caldwell *et al*, 1998; Mancao *et al*, 2005). Therefore, EBV expresses the antigens that are necessary for the survival of its host cell, which would otherwise be destined to apoptose; it also exploits the lack of immune surveillance to express a full complement of antigens – latency III programme – to promote its growth.

T-cells and cytokines are also believed to play a part in tumour outgrowth. Both CD4+ and CD8+ T-cells have been detected in PTLD tumours, though CD4+ T-cells appeared to be more abundant (Perera *et al*, 1998). Perera *et al* (1998) postulated that these infiltrating CD4+ T-cells were contributing to the pathogenesis of the disease. Research on the tumour microenvironment has been based on animal models such as the hu-PBL-SCID mouse (human-peripheral blood lymphocyte-severe combined immunodeficiency disease), in which a human immune system is restored in a B- and T-cell deficient mouse by the injection of human PBMC (Mosier *et al*, 1988). However, when PBMCs from EBV-positive donors were used, the SCID mice developed EBV-positive lymphomas (Mosier *et al*, 1988) and as a result hu-PBL-SCID mice have been used extensively to study lymphomagenesis and in particular PTLD development (Rowe *et al*, 1991). Using the hu-PBL-SCID mouse, Johannessen *et al* (2000) showed that without T-cells (CD4+ T-cells in particular) tumour incidence was considerably reduced. Johannessen *et al* (2000) also put forward a tumourigenesis model in which tumour cells initially required T-cell help by way of cytokine production, with tumour cells then progressing to sustain themselves in a cytokine-dependent autocrine fashion and becoming independent of T-cell help altogether. The cytokines found to be produced by the tumour cells generated in this model were IL-2, IL-4, IL-6, IL-10 and IFN-γ and all of these

cytokines are potential direct and indirect stimulators of B-cell growth (Johannessen *et al*, 2000).

The pathogenesis of PTLD is a complex multi-step process that is dependent on a large number of factors such as T-cells immunosuppression, graft type, T-cell contribution to B-cell growth, accumulation of genetic mutations and also EBV's oncogenic potential. There are undoubtedly other factors, host and viral, that contribute to PTLD pathogenesis, although these have yet to be established.

1.4.2.5 Clinical presentation and prevention

The clinical presentation of PTLD is diverse and may present with a wide range of signs and symptoms, which can include a persistent febrile illness and lymphadenopathy (Crawford, 2001). These signs and symptoms are similar to those seen in acute IM. The highest incidence of PTLD occurs in the first year post-transplant and may involve the graft as well as other sites such as the gastrointestinal tract, the lungs and the central nervous system (Gottschalk *et al*, 2005). As PTLD presentation is diverse (and rather non-specific), much research has gone in the development of reliable prevention/monitoring methods, such as measuring EBV viral loads especially as early detection and diagnosis are associated with better outcome (Gottschalk *et al*, 2005). Many PCR-based methods have been used to measure EBV viral load but no standard "cut-off" or indicator values for the development of PTLD have been established and such methods do have limitations, as PTLD has been seen in patients with no detectable increase in EBV viral load (Anonymous, 2004; Axelrod *et al*, 2003; Straathof *et al*, 2002). Furthermore EBV DNA can be detected in whole blood, plasma, PBMC and oropharyngeal secretions but the levels in each of these secretions differ, such that there is good correlation between whole blood and PBMC but a poor correlation between plasma and PBMC (Wadowsky *et al*, 2003). This lack of correlation has been seen in heart, renal and liver transplant patients diagnosed with PTLD, in whom oropharyngeal shedding of EBV was high but correspondingly high levels were not detected in PBMC (Savoie *et al*, 1994). Whole blood would appear to be the specimen of choice as it includes

all blood fractions and may provide the most information regarding viral load (Stevens *et al*, 2001a). Consensus has been reached with regards to serial monitoring of EBV viral load in transplant patients. 50% of SOT patients present with elevated EBV viral load but it is those whose viral load increases rapidly that are high risk of developing PTLD (Gottschalk *et al*, 2005). This may happen very quickly: EBV viral load has been found to double in 56 hours reaching levels indicative of PTLD development (Stevens *et al*, 2001b). Clearly, serial monitoring is the only way to detect such changes. The benefit of serial monitoring combined with pre-emptive reduction in immunosuppression has been demonstrated in one centre where the incidence of PTLD was reduced from 16% to 2% in paediatric liver transplant recipients (Lee *et al*, 2005).

Recently, a group of researchers infected (with EBV) two EBV-negative individuals who were due to receive EBV-positive grafts, through a series of three EBV-positive blood transfusions (Babel *et al*, 2005). Both patients seroconverted prior to transplant and, at the time of publication, both patients had been free of PTLD for a follow-up period of five years. This may be a simple and promising, though unconventional, way of reducing/eliminating the risk of PTLD related to EBV seronegativity.

1.4.3 Treatment of PTLD

The therapies available for the treatment of PTLD in SOT patients aim to quench tumour growth/kill tumour cells or to restore cellular immunity and in particular EBV-specific cytotoxic T-cell activity. The therapies used for the treatment of PTLD are discussed below. Reduction of immunosuppression aims to restore T-cell activity and both chemotherapy and rituximab treatment aim to tackle tumour growth. When combined with close serial monitoring of EBV viral loads, reduction of immunosuppression may also be used prophylactically, as discussed above.

1.4.3.1 Reduction of immunosuppressive drugs

As the occurrence of PTLD is intrinsically linked with iatrogenic immunosuppression, the first line of treatment is a reduction in the dose of immunosuppressive drugs in order to restore cellular immunity (EBV-specific cytotoxic T-cells). The degree to which immunosuppression is reduced differs in each patient, although in cases where PTLD has been formally diagnosed immunosuppressive drugs should ideally be stopped altogether (Anonymous, 2004), which may not be an option as this carries a high risk of graft-versus-host-disease (GVHD) hence it is a very delicate balancing act, especially where there is no secondary therapy (such as dialysis for renal transplant patients). Remission following reduction in immunosuppression ranges from 25% to 63% in adults and 40% to 86% in paediatric patients (Gottschalk *et al*, 2005; Taylor *et al*, 2005). Response usually occurs within three to four weeks of immunosuppression reduction and persists beyond this initial period (Starzl *et al*, 1984). A good response to a reduction of immunosuppression may be seen in PTLD patients whose tumour cells are expressing all latency III antigens, as may be in most tumours occurring within the first year post-transplant, but where antigen expression is restricted, as may be seen in tumours occurring over one year post-transplant, the response may not be as satisfactory. Mortality is high (50% to 90%) in patients who do not respond to a reduction in immunosuppression (Taylor *et al*, 2005).

1.4.3.2 Chemotherapy

Chemotherapy is usually a second line therapy for PTLD, although a recent report shows that it has been preferentially used in paediatric heart transplant patients presenting with monomorphic lesions across a number of institutions (61% patients treated with chemotherapy and 39% treated by reduction of immunosuppression) (Webber *et al*, 2006). This method of treatment was associated with 75% survival at one year and 65% at five years post-diagnosis. Chemotherapy would otherwise be used in patients who have not responded to a reduction/cessation of immunosuppression or in cases where reduction/cessation of immunosuppression may lead to graft loss (Bollard *et al*, 2003). Mortality associated with chemotherapy

toxicity and infection has been reduced through the use of low-dose chemotherapy and granulocyte-macrophage colony stimulating factor by preventing neutropenia (Davis, 2001). The severity of the side-effects of chemotherapeutic agents was increased in PTLD patients due to profound immunosuppression (Oertel *et al*, 2003). In one study involving such patients, where conventional chemotherapy regimens were used, toxicity-related mortality was 26% and infections saw 52% of patients requiring hospitalisation (Elstrom *et al*, 2006). The overall response rate to chemotherapy was 74% in this study. In another study comprising 36 paediatric SOT patients with PTLD (fulminant n=4 and non-fulminant n=32) who had not responded to immunosuppression reduction/withdrawal, antiviral therapy, surgery, rituximab therapy and interferon-α, treatment with low-dose chemotherapy was implemented: the overall response rate was 83% and two-year overall survival rate was 73% (Gross *et al*, 2005). However, the outcome for patients with fulminant PTLD was poor with all patients dying within two years (Gross *et al*, 2005). So, whilst chemotherapy has improved for the treatment of PTLD, its use remains difficult.

1.4.3.3 Anti-CD20 monoclonal antibody (rituximab) therapy

Previously used anti-B-cell monoclonal antibodies include anti-CD21 and anti-CD24 antibodies and, although these are no longer commercially available (Newstead, 2005), they were shown to be safe and relatively effective in a cohort of 57 PTLD patients with a complete response rate of 61%, a relapse rate of 8% and a survival rate of 46% at 1 year (Benkerrou *et al*, 1998). The focus is now on rituximab, a chimeric mouse/human anti-CD20 monoclonal antibody, which was first licensed in the UK for the treatment of follicular non-HD in March 2002 (NICE, 2006). It has also been used for the treatment of PTLD (Cook *et al*, 1999), as over 90% of tumours are CD20-positive B-cells. Responses to rituximab can range between 60% and 100%, although such response rates have been achieved when rituximab was used in conjunction with other forms of therapy such as immunosuppression reduction, chemotherapy and surgery (Berney *et al*, 2002; Gottschalk *et al*, 2005; Milpied *et al*, 2000; Oertel *et al*, 2005). In a recent study on the efficacy of rituximab monotherapy, in which immunosuppression was maintained throughout the study, the overall

response rate to treatment at 1 year was 34.1% (Choquet *et al*, 2006). This highlights the possibility that the response range given above (60% to 100%) may be an overestimate due to the use of other forms of combined therapy. However, rituximab has been shown to be safe with no direct treatment-related deaths reported, although side-effects such as hypertension, purpura with myalgia and intestinal perforation and infections at the site of PTLD have been documented (Blaes *et al*, 2005; Choquet *et al*, 2006; Elstrom *et al*, 2006).

Whilst rituximab targets CD20-positive B-cells, it does not contribute to restoring EBV-specific T-cell immunity and it has been shown that PTLD may reoccur in patients treated with rituximab (Savoldo *et al*, 2005). It has also been postulated that the ablation of the B-cell compartment may remove essential antigenic stimulation for T-cells, which may in turn hamper the recovery of T-cell mediated immunity. (Bollard *et al*, 2003; Straathof *et al*, 2002). Repeated rituximab treatment has also been associated with a loss of CD20 expression on non-HD tumour cells, thus making these tumour cells refractory to further treatment by this means (Davis *et al*, 1999). Whilst this has not been documented in the context of PTLD, it may occur nonetheless and be responsible for the non-response of some patients to multiple courses of rituximab.

1.4.3.4 Other therapies for PTLD

Other therapies that have been used for the treatment of PTLD include surgical resection, radiotherapy and the use of anti-IL-6 monoclonal antibodies and IFN-α. Each of these forms of treatment has been implemented either concurrently with a reduction of immunosuppression or following a failure to respond to a reduction of immunosuppression. Treatment of PTLD by surgical resection has been successful in patients with clearly localised tumours, as has radiotherapy (Foroncewicz *et al*, 2006; Newstead, 2005). Such treatment is of clear benefit where the tumour is compressing and compromising vital organs (Gottschalk *et al*, 2005).

Immunotherapeutic methods using IFN-α and anti-IL-6 antibody have both been used with varying success. IFN-α was used as it stimulates cytotoxic T-cell and NK cell activity and IL-6 was targeted as it is critical in promoting B-cell growth and hence tumour growth. A 57% (8 out of 14 patients) response rate was achieved using IFN-α in one study, although side effects including flu-like symptoms and neutropenia were described and approximately 30% (4 out of 14 patients) of patients presented with signs of graft rejection, which was treatable using steroids but was dependent on the degree of immunosuppression used (Davis, 2001; Davis *et al*, 1998). Five of the eight patients who responded to IFN-α treatment subsequently died due to infection and two required further chemotherapy. A study by Haddad *et al* (2001) showed that the use of a murine monoclonal anti-IL-6 antibody achieved complete remission in five, and partial remission in three out of 12 SOT PTLD patients, whose PLTD was refractory to reduction in immunosuppression, without relapse at 15 to 27 months follow-up. There were no severe side effects associated with the use of monoclonal anti-IL-6 antibody (Haddad *et al*, 2001). Whilst these immunotherapeutic methods demonstrated a degree of success, no controlled trial was carried out and the studies described here were small.

Antiviral agents, such as aciclovir, ganciclovir and valaciclovir, have also been used, especially to prevent PTLD in EBV seronegative recipients of EBV positive grafts (Malouf *et al*, 2002). In the majority of cases "donor EBV" from the graft infects the EBV-negative recipient's B-cells, in which lytic replication of EBV takes place. This can give rise to EBV infection, which may result in PTLD in cells of recipient origin but following primary infection by graft donor EBV (Haque *et al*, 1996). The period of primary infection of recipient B-cells may therefore be a useful time window in which to administer antiviral agent. These antiviral agents only suppress lytic replication of EBV and not latent replication, which is the source of PTLD development, although evidence of lytic reactivation has been detected in up to 80% of tumours with 30% to 40% of tumours positive for the EBV lytic antigen and structural glycoprotein gp350 (Tanner and Alfieri, 2001). Other reports indicate that only approximately 1% of EBV-infected B-cells undergo lytic replication at any one

time and therefore these agents may only be of limited use (Newstead, 2005). A recent report by Malouf *et al* (2002) suggested that antiviral therapy significantly reduced PTLD in lung and heart/lung transplant patients: none of their EBV seronegative patients on antiviral therapy developed PTLD (15 patients – although the patients were not receiving anti-T-cell antibodies, a factor which may have improved their prognosis) (Malouf *et al*, 2002). These data concur with earlier reports of PTLD incidence of 0.5% in a large cohort (198 patients) who received prophylactic antiviral therapy even whilst receiving anti-T-cell therapy (OKT3, anti-thymocyte globulin and Minnesota antilymphocyte globulin) (Darenkov *et al*, 1997), which has been associated with an increase in PTLD (Burns and Crawford, 2004; Darenkov *et al*, 1997). Other research groups have sought to render EBV-infected cells sensitive to ganciclovir by inducing the expression of viral thymidine kinase (*in vivo* in the context of a clinical trial in phase I/II) (Faller *et al*, 2001) and by inducing lytic replication using chemotherapy (*in vitro* in epithelial cells) in one case (Feng *et al*, 2002). Another important factor to bear in mind is that some these antiviral agents will also be effective against CMV, which has been postulated as a PTLD risk factor (Newstead, 2005).

1.4.3.5 Summary of PTLD therapies

All of the therapies discussed above have demonstrated variable levels of success over time. Each of these therapies is associated with side-effects ranging from flu-like symptoms to neutropenia and in some cases death due to treatment toxicity or infection. Additionally, some of these therapies may not be effective in part due to the therapy itself but also due to patients being highly immunosuppressed and/or presenting with advanced or progressive PTLD disease. Central to of all these considerations is the preservation of the graft, which may be compromised when reducing immunosuppression or using IFN-α due to GVHD. A novel approach to tackling this therapeutic conundrum has been the development of adoptive cellular immunotherapy, which has shown promising results without significant side-effects or the risk of graft loss.

1.5 Adoptive immunotherapy

Adoptive T-cell immunotherapy is a novel form of therapy for the treatment of PTLD, which was developed to overcome the shortcomings and side-effects of all other conventional PTLD therapies. The aim of adoptive T-cell immunotherapy is to restore EBV-specific T-cell activity in transplant recipients, with a view to targeting EBV positive B-cell tumours.

1.5.1 Development of adoptive immunotherapy

1.5.1.1 Rationale and development of adoptive immunotherapy

T-cells are at the centre of immunological function and these are essential in the control of latent and persistent viruses such as HIV, CMV and EBV. The T-cell response that controls EBV is described in section 1.3.2. However, this T-cell response is iatrogenically suppressed after organ transplantation and it can also be suppressed by HIV infection. Both EBV and CMV can give rise to severe disease in organ transplant patients (as described above for EBV). HIV leads to progressive destruction of CD4+ T-cells and incidentally profound immunosuppression. Based on the understanding of these diseases and their interaction with the immune system, it was postulated that restoring T-cell immunity by adoptive immunotherapy would overcome PTLD progression. Early work into development of immunotherapy for HIV, CMV and EBV was undertaken in a variety of animal models but in 1992 Riddell *et al* were the first to restore CMV-specific T-cell immunity in three BMT patients by adoptively transferring CMV-specific CTL clones generated from their CMV-positive BMT donors. None of the three patients developed of CMV pneumonitis (Riddell *et al*, 1992). In 1994, Papadopoulos *et al* used bone marrow donor PBMCs to treat five recipients who had developed EBV-associated PTLD of donor origin. Three of the five patients treated responded well and remained disease-free up to 16 months post-transplant, though all three patients developed GVHD (Papadopoulos *et al*, 1994). Two patients died of pulmonary complications not

thought to be attributable to treatment (Papadopoulos *et al*, 1994). Some investigators progressed from PBMCs to purified T-cells but with limited success and high rates of GVHD due to the presence of alloreactive T-cells (Heslop *et al*, 2004). With an increasing understanding of EBV viral immunology and PTLD, investigators have moved on to develop novel immunotherapeutic methods using autologous and allogeneic EBV-specific CTLs for the treatment of PTLD. PTLD is an "ideal candidate" disease for treatment by immunotherapy as PTLD tumours are highly immunogenic and in most cases express all EBV latent antigens (latency III), which are targets for CTLs.

1.5.1.2 Adoptive immunotherapy in bone marrow transplant patients

One year on from Papadopoulos *et al*'s (1994) treatment of BMT patients with PTLD using PBMCs, Rooney *et al* (1995) infused 10 BMT patients (of whom seven had received HLA-matched donations from unrelated donors and three had received HLA-mismatched donation from family members) with donor-derived polyclonal EBV-specific CTLs. Of the 10 patients included in this controlled trial, three patients had signs of uncontrolled EBV replication (presenting as elevated EBV DNA viral load or PTLD) and seven were infused prophylactically. All three patients who presented with uncontrolled EBV replication responded to treatment with falling EBV viral loads, additionally none developed CTL-induced GVHD (Rooney *et al*, 1995). None of the seven patients infused prophylactically developed PTLD. The CTLs infused in the patients had been genetically modified with a neomycin resistance gene so that they could be traced post-infusion. The CTLs could be detected up to 10 weeks post-infusion (Rooney *et al*, 1995). The generation of donor-derived EBV-specific CTLs using PBMCs overcame the problems of alloractivity and GVHD previously encountered with PBMC.

A further study was carried out by Rooney *et al* (1998) involving 100 patients: 39 received CTLs (generated as above) and 61 did not receive CTLs and acted as historical controls. The patients who received the CTLs were considered to be at high risk of PTLD following allogeneic BMT. Of the 39 patients who received autologous

CTLs none developed PTLD, whereas 11.5% of patients in the control group presented with PTLD (Rooney *et al*, 1998b). Two patients who presented with PTLD but who had not received prophylactic CTLs were treated successfully with CTL infusions as part of the above study (Rooney *et al*, 1998b). Another patient who was treated using the same method did not respond to CTL treatment and succumbed to PTLD (Gottschalk *et al*, 2001). It emerged that this patient harboured more than one EBV strain: one strain with wild-type EBNA-3B and one strain with an EBNA-3B deletion. Prior to the transplant only the wild-type EBNA-3B virus could be detected in both the donor and the recipient but after transplantation both strains could be detected in the recipient. After CTL infusion only the EBNA-3B deletion mutant strain was detectable indicating that the CTLs had selected out this resistant strain, as the CTLs generated from donor PBMCs were only reactive to wild-type EBNA-3B (Gottschalk *et al*, 2001). Such treatment escape mutants were found in one of four patients treated by Rooney *et al* (1998) (Gottschalk *et al*, 2001) and may therefore contribute to some of the treatment failures observed. Whilst treatment failure has been documented, adoptive T-cell immunotherapy has clearly been shown to be safe and effective as long-term prophylaxis and/or treatment for PTLD following BMT as these genetically modified CTLs have been detected up to 78 months post-infusion (Bollard *et al*, 2004).

1.5.1.3 Adoptive immunotherapy in solid organ transplant patients

The situation in SOT differs somewhat from BMT, as PTLD tumours are usually of recipient origin and therefore the use of donor cells would not be appropriate (especially as the majority of grafts will be of cadaveric origin). PTLD tumours in EBV-naïve recipients may also be of recipient origin as EBV infection of recipient B-cells occurs through the transfer of EBV via the grafted organ (Haque *et al*, 1996). So instead of generating CTLs from the graft donor as in BMT, autologous (to the recipient) and allogeneic CTLs have been developed for the treatment of PTLD in SOT.

Autologous immunotherapy for the treatment of PTLD in SOT also initially involved the use of PBMCs (Todo *et al*, 1995) and lymphokine-activated killer (LAK) cells supplemented with IL-2 (Nalesnik *et al*, 1997). However, neither of these approaches have been developed further and much like the progression of treatment seen in BMT, the generation of EBV-specific CTL using autologous PBMCs was favoured (Comoli *et al*, 2002; Haque *et al*, 1998; Khanna *et al*, 1999; Sherritt *et al*, 2003). The CTLs generated were polyclonal, as these would recognise a number of EBV epitopes. The CTLs generated by these investigators were used not only prophylactically (Comoli *et al*, 2002; Haque *et al*, 1998) but were also used for the treatment of PTLD (Khanna *et al*, 1999; Sherritt *et al*, 2003). The three patients infused prophylactically by Haque *et al* (1998) all showed an increase in CTL precursor (CTLp) frequency that in turn increased after each subsequent monthly infusion (3 infusions were administered) concurrent with a decrease in EBV DNA viral load. The CTLp were detected up to three months after the final infusion. The seven patients treated by Comoli *et al* (2002) all also showed an increase in CTLp frequency, although only five of the seven patients had a decrease in EBV DNA viral load. None of the patients treated during the course of both studies developed PTLD. Both PTLD patients treated by Khanna *et al* (1999) and Sherritt *et al* (2003) responded to initial CTL infusions and to subsequent infusions following relapse, however one patient died of pulmonary vein necrosis and haemorrhage (Khanna *et al*, 1999). Such complications had been previously described by Rooney *et al* (1998) in a patient with bulky disease who required ventilation following the administration of CTLs. A point common to all four of these studies is that the infused CTLs could only be detected up to three months post-infusion which was in stark contrast to CTLs infused in BMT patients that can be detected up to 78 months post-infusion (Bollard *et al*, 2004). The reduced survival time of infused CTLs in SOT is highly likely to be associated with the permanent immunosuppression required for allograft maintenance in SOT patients.

When adoptive CTL immunotherapy was being developed it was believed that it would not be possible to generate EBV-specific CTLs from recipients who were

EBV-negative at the time of transplant and who were also profoundly immunosuppressed (Khanna *et al*, 1999). However, this was not the case as a number of investigators succeeded using a variety of methods (Comoli *et al*, 2002; Comoli *et al*, 2005; Khanna *et al*, 1999; Metes *et al*, 2000). Another technical consideration was the potential effect of immunosuppression on the B-cell compartment, with regard to growth of LCLs *in vitro*, as these are vital in the generation of EBV-specific CTLs. A study by Savoldo *et al* (2001) found that there was a median delay of 11 days in the establishment of LCLs from immunosuppressed patients compared to those of healthy controls, however no damage to the B-cells was noted (Savoldo *et al*, 2001). The mechanism underlying this delay was not established but it increases the overall time taken for the generation of CTLs, which could be critical as PTLD can be aggressive and rapidly progressing. The time for LCL generation was estimated to range from four to six weeks, followed by another four to six weeks for the generation of CTLs (Savoldo *et al*, 2000), although this may be longer depending on the number of cells required. The CTL generation time is probably the greatest drawback of autologous adoptive immunotherapy. There is also great disparity in the approach to the generation of CTLs as Haque *et al* (1998) cultured CTLs pre-transplant and cryopreserved these cells until required, whereas both Khanna *et al* (1999) and Comoli *et al* (2002) generated CTLs using PBMCs from SOT patients who had either seroconverted post-transplant (Khanna *et al*, 1999) or who were presenting with elevated EBV viral loads (Comoli *et al*, 2002). The development of autologous EBV-specific CTLs is time consuming, and also expensive, especially in cases where CTLs are generated prior to transplant and not required by the patient. In order to overcome these hurdles Dr Tanzina Haque and colleagues in our laboratory sought to develop a bank of allogeneic HLA-typed EBV-specific polyclonal CTLs generated from healthy blood donors, from which CTLs would be used to treat PTLD patients based on a best HLA-matched basis as discussed below.

1.5.2 Cytotoxic T-cell bank

The development of a CTL bank would provide an easily accessible resource for the treatment of PTLD on a best HLA-matched basis, which would overcome the time and technical constraints surrounding the generation of autologous CTLs. To this end a bank of more than 100 CTLs have been generated by Dr Haque and colleagues. Cytotoxic T-cell lines were grown for up to 10 weeks in order to generate sufficient CTLs and these were subsequently cryopreserved until required. All cell lines were microbiologically tested, and their cytotoxicity and extracellular phenotype assessed as described by Wilkie *et al* (2004). Another advantage of such a cell bank is that a wide spectrum of HLA-types could be covered, therefore increasing the likelihood of having suitable CTLs for a wide variety of patients. Human leucocyte antigen-matching (HLA) was based on MHC class I matching with at least one HLA-A match and one HLA-B match and where possible MHC class II (HLA-DR) was also matched (Wilkie *et al*, 2004). The effectiveness of the CTLs was assessed by *in vitro* cytotoxicity assays using as targets patients' phytohaemagglutinin (PHA) blasts and LCLs (where available), autologous PHA blasts and LCLs, HLA-mismatched LCLs and K562 cells that are sensitive to NK cell killing.

CTLs generated as part of the bank described by Wilkie *et al* (2004) have been infused in one bowel and liver transplant patient leading to complete regression of PTLD with CTLp detectable up to 11 weeks post-infusion (Haque *et al*, 2001) and in a further eight patients (who had not responded to other treatments) in the context of a phase I/II clinical trial, with five patients completing treatment (Haque *et al*, 2002). Of the three patients who did not complete the course, two died of causes unrelated to CTL infusions and one patient received two infusions and then declined further treatment. Of the five patients who completed treatment, three showed complete response with tumour regression and improved graft function and two did not respond, possibly due to widespread disease. The patients who responded had been free of disease from 11 months to 28 months at the time of publication. No side-effects or toxic effects and no GVHD were noted following the CTL infusions. Whilst this was a small-scale trial, it demonstrated the fact that adoptive

immunotherapy using partly HLA-matched allogeneic CTLs from a cryopreserved bank of CTLs for the prompt treatment of PTLD was feasible, functional and useful. The encouraging results of the trial conducted by Haque *et al* (2002) were further supported when CTLs from the bank were used to treat an EBV-positive primary B-cell lymphoma in the central nervous system in a case of primary immunodeficiency (Wynn *et al*, 2005). The young girl concerned received cytotoxic and antiviral chemotherapy but remained in a comatose state. However, she achieved a full recovery with immunotherapy with partly HLA-matched allogeneic CTLs following which, her immunodeficiency was corrected by a non-T-cell depleted peripheral stem cell transplant from a matched unrelated donor (Wynn *et al*, 2005). This case showed that CTLs were able to cross the blood-brain barrier and effect their cytotoxic potential unhindered and overcame the question of whether concomitant reduction of immunosuppression in the PTLD patients was the cause of their tumour regression.

Whilst CTLs generated as part of the cryopreserved bank established in our laboratory have clearly been shown to be effective *in vivo*, little is known about their development *in vitro*. It may be that a greater understanding of CTL development *in vitro* would enable a wider and more effective use of such cells *in vivo*.

1.5.3 Adoptive immunotherapy for other EBV-associated malignancies

Cytotoxic T-cells have been used for the treatment of other EBV-associated malignancies such as HD and NPC, as both NPC tumours and HD tumours express latency II EBV antigens. Autologous CTLs were generated using PBMCs from patients with NPC (Straathof *et al*, 2005) and HD (Rooney *et al*, 1998a; Roskrow *et al*, 1998) but allogeneic CTLs were also generated for the treatment of HD using healthy EBV-positive blood donors (Lucas *et al*, 2004). These cytotoxic T-cells have been used safely and relatively successfully in the treatment of NPC (Straathof *et al*, 2005) and HD (Lucas *et al*, 2004; Rooney *et al*, 1998a; Roskrow *et al*, 1998). LCLs were used as antigen presenting cells for the generation of both HD CTLs (Lucas *et*

al, 2004; Roskrow *et al*, 1998) and NPC CTLs (Straathof *et al*, 2005) although LMP-1- (Gottschalk *et al*, 2003) and LMP-2A- (Gahn *et al*, 2001; Rooney *et al*, 1998a; Su *et al*, 2001) transduced DCs have also been used as antigen presenting cells. LMP-1 and LMP-2 are poorly immunogenic in comparison to the EBNA-3 family antigens to which CTLs predominantly respond following *in vitro* culture with LCLs, which may be why more specific methods of CTL growth were developed using other cells besides LCL.

Until recently it was believed that EBNA-1 was in effect "immunologically invisible" to CD8+ T-cells as it contains a glycine-alanine repeat that prevents its proteasomal degradation (Levitskaya *et al*, 1995; Levitskaya *et al*, 1997) but also prevents its translation (Yin *et al*, 2003). However it has now been reported that EBNA-1 can be presented via MHC class I and recognised by CD8+ T-cells (Lee *et al*, 2004; Voo *et al*, 2004). These CD8+ T-cells could recognise EBNA-1 expressed by LCLs. In future it may therefore be possible to generate CTLs specific for LMP-1, LMP-2 but also EBNA-1. If EBNA-1-specific CTLs could be generated and expanded *in vitro* this could potentially be of great benefit in BL where tumour cells only express EBNA-1, but also in other malignancies. Genetic modifications of both T-cells and antigen presenting cells have also been carried out to improve LMP-1 and LMP-2 recognition. LMP-1 has toxic effects on antigen presenting cells hindering the development of LMP-1-specific cytotoxic T-cells. A non-toxic non-functional deletion mutant form of LMP-1 was generated by Gottshalk *et al* (2003) and successfully used to generate LMP-1-specific cytotoxic T-cells. LMP-1-specific CTLs would be of particular interest for the treatment of HD, where LMP-1 is one of the principal antigens expressed. Genetic modification of PBMCs and subsequent generation of effective cytotoxic T-cell has also been developed. The transduction of TCRs from LMP-2-specific clones into PBMCs gave rise to cytotoxic T-cells capable of lysing LCLs expressing LMP-2 (Jurgens *et al*, 2006). The generation of LMP-2 specific cytotoxic T-cells would also be of clinical interest in the treatment of HD and NPC.

1.6 Aim

PTLD has been successfully treated with cellular adoptive immunotherapy using EBV-specific CTLs. However, little is known about the development of these CTLs. This project therefore sought to establish how the CTLs developed in terms of their growth, phenotype and T-cell receptor (TCR) repertoire diversity. It also sought to establish which cytolytic molecules were present in the CTLs and which pathways they employed to effect their cytotoxic potential. It is hoped that this basic characterisation of CTLs will reveal features that can potentially enhance the clinical effects of CTLs.

Chapter 2

Materials and methods

2.1 Details of suppliers and manufacturers

2.2 Tissue culture methods

2.3 Molecular methods

2.4 Fluorescence associated cell sorting

2.5 Western blotting

2.1 Suppliers and manufacturers

Amersham Biosciences	Amersham Place, Little Chalfont, HP7 9NA, UK
Applied Biosystems	7 Kingsland Grange, Woolston, Warrington, WA1 4SR, UK
BD Biosciences	21 Between Towns Road, Cowley, Oxford, OX4 3LY, UK
BD PharMingen	21 Between Towns Road, Cowley, Oxford, OX4 3LY, UK
BDH (VWR Intl Ltd)	Merck House, Poole, Dorset, BH15 1TD, UK
Beckman Coulter	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
Becton Dickinson	21 Between Towns Road, Cowley, Oxford, OX4 3LY, UK
Biometra GmbH i. L.	Rudolf-Wissell-Straβe 30, 37079 Goettingen, Germany
Caltag Medsystems Ltd	PO Box 6139, Silverstone, Towcester, NN12 8UP, UK
Chiron Corporation	4560 Horton Street, Emeryville, 94608-2926 California, USA
Dynal Biotech ASA	P.O. Box 114 Smestad, N-0309, Oslo, Norway
ECACC	Porton Down, Wiltshire, SP4 0JG, UK
Fisher Scientific UK Ltd	Bishop Meadow Rd, Loughborough, Leicestershire, LE11 5RG, UK
Greiner BioOne Inc	1205 Sarah St, Longwood, 32750 Florida, USA
Hanna Instruments Ltd	Eden Way, Pages Park Industrial Estate, Leighton Buzzard, LU7 4AD, UK
Harlan Sera-Lab	Dodgeford Lane, Loughborough, LE12 9TE, UK

Invitrogen	3 Fountain Drive, Inchinnan, Business Park, Paisley, PA4 9RF, UK
ISP Europe	Waterfield, Tadworth, Surrey, KT20 5HQ, UK
Miltenyi Biotec Ltd	Almac House, Church Lane, Bisley, Surrey, GU24 9DR, UK
MDS Nordion	447 March Road, Ottawa, Ontario, K2K 1X8, Canada
MP Biomedicals, LLC	5th floor, 86 Jermyn Street, London, SW1Y 6AW, UK
Nunc	Nalge (Europe) Ltd, Unit 1a, Thorn Business Park, Hereford, HR2 6JT, UK
Oxoid Limited	Wade Road, Basingstoke, RG24 8PW, UK
Perkin Elmer Life Sciences	Chalfont Road, Seer Green, Beaconsfield, HP9 2FX, UK
Pharmacia Biotech	Acquired by Amersham Biosciences, Amersham Place, Little Chalfont, HP7 9NA, UK
Promega UK	Delta House, Chilworth Science Park, Southampton, SO16 7NS, UK
Qiagen Ltd	Qiagen House, Fleming Way, Crawley, RH10 9NQ, UK
R&D Systems	19 Barnton Lane, Abingdon Science Park, Abingdon, Oxon, OX14 3NB, UK
Roche	Roche Diagnostics Ltd, Bell Lane, Lewes, BN7 1LG, UK
Sandoz Ltd	Unit 37, Woolmer Way, Bordon, GU35 9QE, UK
Sanyo Gallenkamp (MSE)	Monarch Way, Belton Park, Loughborough, LE11 5XG, UK
Sigma	Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, SP8 4XT, UK

Sigma-Genosys Ltd	Sigma-Aldrich House, Homefield Business Park, Homefield Road, Haverhill, CB9 8QP
SLS	Coatbridge Business Centre, 204 Main Street, Coatbridge, ML5 3RB, UK
StemCell Technologies	777 West Broadway, Suite 808, Vancouver, V5Z 4J7, BC, Canada
Stratagene	11011 N. Torrey Pines Road, La Jolla, California CA92307, USA
Sterilin	Barloworld Scientific Ltd. Beacon Road, Stone, ST15 0SA, UK
Stuart Scientific	Barloworld Scientific Ltd. Beacon Road, Stone, Staffordshire, ST15 0SA, UK
Thermo	The Ringway Centre, Edison Road, Unit 5, Basingstoke, UK
Ultra-Violet Products Ltd	Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge, CB4 1TG, UK
VWR International Ltd	Merck House, Poole, BH15 1TD, UK

2.2. Tissue culture methods

2.2.1 Tissue culture consumables, chemicals, media and equipment

2.2.1.1 Tissue culture consumables

Tissue culture flasks, 96-well U-bottomed plates, 48-well plates and cryovials were supplied by Nunc (Nalge Ltd, Hereford, UK). All other plasticware was supplied by Greiner-Bione (Leighton Buzzard, UK), SLS (Coatbridge, UK) and Sterilin (Stone, UK). LP-2 γ-counter tubes were supplied by Thermo (Basingstoke, UK).

2.2.1.2 Chemicals and media

All media used in this section are detailed in table 2.1.

Media names	Medium	L-Glut	P/S	NEAA	FBS	BrdU
10% RPMI	RPMI	2mM	100IU/ml	X	10% (v/v)	X
20% RPMI	RPMI	2mM	100IU/ml	X	20% (v/v)	X
10% DMEM with BrdU	DMEM	2mM	100IU/ml	1% (v/v)	10% (v/v)	15μg/ml
10% DMEM	DMEM	2mM	100IU/ml	1% (v/v)	10% (v/v)	X
DC medium	RPMI	2mM	100IU/ml	X	10% (v/v)	X
HBSS	HBSS	X	X	X	X	X
RPMI	RPMI	X	X	X	X	X
Supplier	Invitrogen	Invitrogen	Invitrogen	Sigma	Invitrogen Harlan	Sigma

Table 2.1: Tissue culture media used (Invitrogen, Paisley, UK; Sigma, Gillingham, UK and Harlan, Loughborough, UK). Abbreviations used: Rosewell Park Memorial Institute (RPMI), Dulbecco's Modified Essential Medium (DMEM), Dendritic cell medium (DC medium), Hank's Balanced Salt Solution (HBSS), L-Glutamine (L-Glut), Penicillin/Steptomycin (P/S), Non-Essential Amino Acids (NEAA), Foetal Bovine Serum (FBS), Bromodeoxyrudine (BrdU).

Histopaque®-1077 was purchased from Sigma and unless otherwise stated, all chemicals were purchased from Sigma. Radioactive chromium - Chromium-51 (^{51}Cr) - was purchased from Amersham Biosciences (Little Chalfont, UK) as sodium chromate in sterile sodium chloride solution at 37MBq/ml (MBq – Mega Becquerels) or 1mCi/ml (Ci – Curie, ml - millilitre). The specific activity of the chromium was >9.25GBq (Giga Becquerels)/mg of chromium or >250mCi/mg of chromium. Sterile Phosphate Buffered Saline (SPBS) (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4 in dH_2O, made to pH7.4 and autoclaved for 15 minutes at 121°C) was made in-house by Ms Deborah Allen, The University of Edinburgh, RDSVS, Veterinary Pathology, Summerhall, Edinburgh, UK.

2.2.1.3 Equipment used

All centrifugation steps were carried out using a Sanyo MSE Mistral 3000E (Rotor 43124-129) or a temperature controlled Sanyo MSE Mistral 3000i centrifuge (Rotor 43124-129) (Loughborough, UK). Centrifugation steps, where ^{51}Cr was involved, were carried out using a Beckman Coulter TJ-6 centrifuge (High Wycombe, UK). Counts per minute (cpm) of γ-radiation released by chromium-labelled target cells were measured using a Perkin Elmer 1480 Wizard 3" Automatic Gamma Counter (Perkin Elmer, Beaconsfield, UK).

2.2.2 Cell culture – PBMC, CTL and LCL

2.2.2.1 PBMC isolation and HLA-typing

PBMCs were isolated from buffy coat fractions of whole blood taken from healthy blood donors at the Scottish National Blood Transfusion Services, Edinburgh, UK (SNBTS). The HLA class I and class II types of the donors were determined by serology and the polymerase chain reaction (PCR) respectively and this was done by the SNBTS. Buffy coats were diluted 1:2 in HBSS at room temperature and subsequently overlayed on an equal volume of Histopaque®-1077 and centrifuged for 20 minutes at 1200g at 20°C. The PBMC interface was collected and transferred

to 10ml to 20ml of 10% RPMI. Numbers and viability of PBMC were established by trypan blue exclusion as described below.

2.2.2.2 Cell counts by trypan blue exclusion

Cells were counted using the trypan blue exclusion method, where a 1:1 mixture of cell suspension and 0.4% trypan blue (MP Biomedicals and Sigma) was applied to a haemocytometer and both the live (unstained) and dead cells (stained) were counted in the 25 central squares. This number was multiplied by the dilution factor of cells and trypan blue and multiplied by 10^4 to give the number of cells per ml of cell suspension. Where the volume of cells added to trypan blue was not in a 1:1 ratio the dilution factor was modified accordingly for the calculations.

2.2.2.3 LCL establishment and maintenance

EBV-immortalised LCLs used were initially setup and grown by the Cancer Research UK group, The University of Edinburgh, Laboratory for Clinical and Molecular Virology, Summerhall, Edinburgh, UK. Briefly, LCLs were established by mixing a loosened cell pellet of $2x10^6$ PBMC to 100 microlitres (µl) of in-house EBV B958 virus preparation (EBV B958 virus preparation was carried out by the Cancer Research UK group) and 10µl of 100µg/ml cyclosporine A (Sandoz). The total volume was made up to 1ml with 10% RPMI and transferred in one well of a 48-well plate. PBMCs were incubated at 37°C in a humidified 5% CO_2 atmosphere. Cultures were fed weekly, split when required and cells transferred to tissue culture flasks upon establishment of the culture. LCLs were transferred to new tissue culture flasks at least once a month.

2.2.2.4 LCL γ-irradiation

LCLs were γ-irradiated with 40 Grays using a Gammacell 3000 Elan irradiator as source of ^{137}Caesium (MDS Nordion). An irradiation indicator sticker was used at each irridation to ensure that the LCLs had been subjected to sufficient radiation (ISP Europe, Tadworth, Surrey, UK).

2.2.2.5 EBV-specific CTL establishment and maintenance

EBV-specific CTLs were generated using fresh or cryopreserved PBMCs isolated from buffy coats and corresponding autologous LCLs following the method described by *. Briefly, γ-irradiated autologous LCLs were used to stimulate PBMCs at a 40:1 PBMC:LCL ratio. The PBMCs and LCLs at 40:1 ratio were resuspended at a concentration of $1x10^6$ cells/ml in 20% RPMI. 10 days after initial stimulation, CTLs were re-stimulated using γ-irradiated autologous LCLs at a 4:1 CTL:LCL ratio. 20IU/ml of recombinant interleukin-2 (IL-2) (Chiron, USA) were added to the culture 14 days after the initial stimulation and every 3 days thereafter. At each weekly stimulation, the CTL concentration was adjusted to $1x10^6$ cells/ml and the CTL were transferred to new tissue culture flask.

2.2.2.6 Cryopreservation of cells

The same cryopreservation protocol was observed for all cell types. Briefly, all cells were centrifuged into a pellet and the supernatant discarded. The cell pellet was loosened and resuspended in 1ml to 1.5ml of freezing medium (10% v/v Dimethyl sulphomethoxasol (DMSO), 90% v/v FBS) and transferred to 1.8ml cryovials. Once in cryovials, cells were stored at -80°C and then transferred to liquid nitrogen for long-term storage.

2.2.2.7 Recovery of cryopreserved cells

Cells were thawed in a 37°C waterbath and transferred dropwise to 25ml of warm wash medium (HBSS or RPMI). Where CTLs were thawed these were added dropwise to 25ml of warm 10% RPMI. The cells were centrifuged at 300g for 7 minutes at 20°C and the supernatant discarded. The cells were resuspended in 10ml to 30ml of 20% RPMI for CTLs and 10% RPMI for all other cell types and subsequently counted by the trypan blue exclusion method (as described in section 2.2.2.2)

2.2.3 Chromium release cytotoxicity assays

2.2.3.1 Standard 4-hour cytotoxicity assay

a) Target cells

$0.5x10^6$ target cells – autologous LCL, HLA-mismatched LCL, K562 cells (sensitive to NK cell activity) – were centrifuged at 300g for 7 minutes and the supernatant was discarded. The target cell pellets were resuspended in the residual supernatant. 50μCi ^{51}Cr were added to each target cell type and these were incubated for 1 hour at 37°C (the volume of ^{51}Cr corresponding to 50μCi was determined at each assay due to the daily decay of chromium – see chapter 7 appendix A2). The target cells were washed twice with 12ml of RPMI or HBSS and centrifuged at 300g for 5 minutes. The target cells were then resuspended in a final volume of 5ml 20% RPMI to give a final concentration of $1x10^5$ cells/ml.

b) Effector cytotoxic T cells

Cytotoxic T cells were resuspended in 20% RPMI at $2x10^6$cells/ml with 0.6ml of CTL suspension required per target cell triplicate. The 96-well U-bottomed plates used in these assays were setup as shown in figure 2.1 with all the CTL/target ratios tested in triplicate.

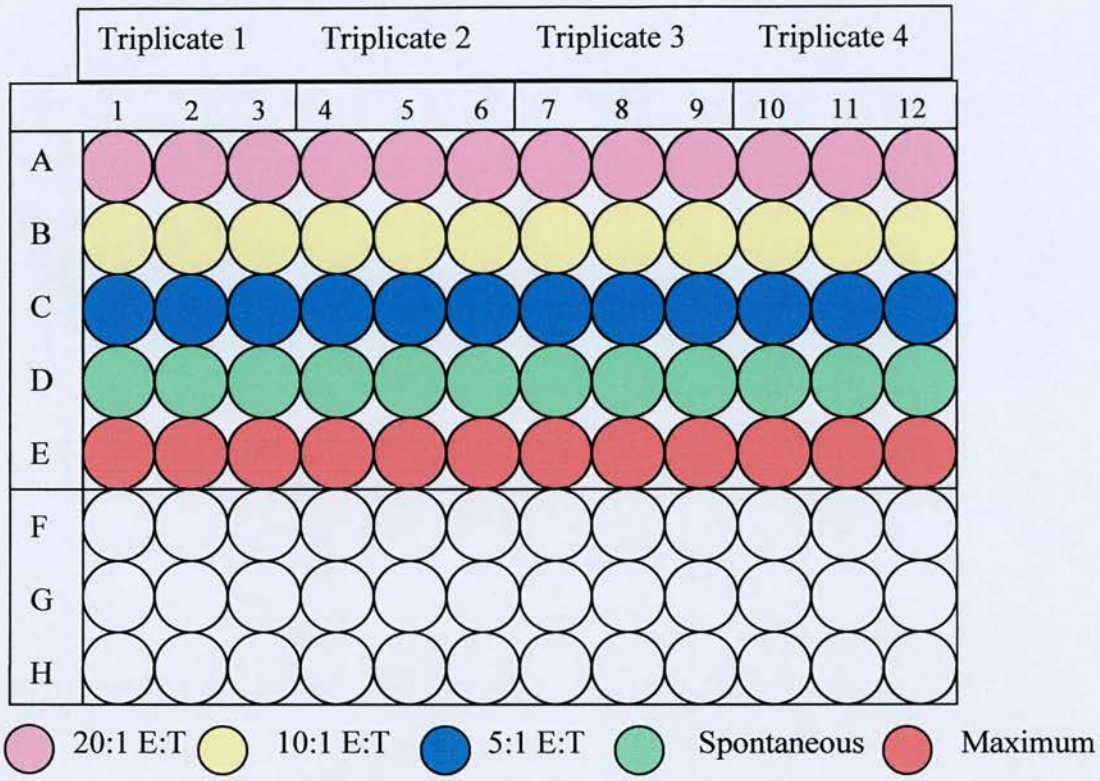


Figure 2.1: Set up of 96-well plate for 4-hour chromium-release assay.

200µl of CTL suspension at $2x10^6$ cells/ml was added to each pink well of a 96-well U bottomed plate. 100µl of 20% RPMI were added to all the wells in rows B (yellow row) and C (blue row). The CTL suspension was diluted 2-fold by taking 100µl of CTL suspension from row A and mixing it with 20% RPMI in row B. This was diluted 2-fold again by taking 100µl from row B and adding it to row C. 100µl from row C were discarded. The final volume of cell suspension in all wells at each dilution was 100µl and the final CTL concentration was $2x10^6$ cells/ml in row A, $1x10^6$ cells/ml in row B and $0.5x10^6$ cells/ml row C.

c) Controls

100µl of 20% RPMI only were added to wells in row D (green wells) and a 100µl of 1% v/v Triton® X-100 were added to wells in row E (red wells). Row D represented

the spontaneous release of chromium by the targets cells and row E represented the maximum release of chromium by the targets cells over the 4-hour period.

d) 4-hour incubation setup

100µl of chromium-labelled targets at $1x10^5$ cells/ml were added to all the wells of the appropriate triplicate. Thus, the effector:target ratio in row A was 20:1, in row B 10:1 and row C 5:1. The effector and target cells were incubated for 4 hours at 37°C. Thereafter, the 96-well plate was centrifuged for 5 minutes at 300g. 100µl of supernatant was collected from each well and transferred to LP2 γ-counter tubes. The tubes were loaded into γ-counter racks and the chromium released was measured using the automatic gamma counter. The percentage of specific cell lysis was measured using the following formula:

$$\% \text{ cell specific lysis} = \frac{(\text{cpm test release} - \text{cpm spontaneous release})}{(\text{cpm maximum release} - \text{cpm spontaneous release})} \times 100$$

for example: $$33.3\% \text{ cells lysis} = \frac{(1229.9 \text{ cpm} - 459.6 \text{ cpm})}{(2772.5 \text{ cpm} - 459.6 \text{ cpm})} \times 100$$

2.2.3.2 4-hour cytotoxicity assay using concanamycin A, brefeldin A, ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid

a) Preparation of CMA, BFA and EGTA

Concanamycin A (CMA) was dissolved in DMSO to a concentration of 100µM and stored at -20°C. Brefeldin A (BFA) was dissolved in 100% biochemical grade ethanol (BDH, Poole, UK) to a concentration of 40mM and also stored at -20°C. Both CMA and BFA were stored in small aliquots to avoid multiple freeze/thawing cycles. Ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was dissolved in distilled water to a concentration of 300mM with the addition of sodium hydroxide to increase its pH and solubility. The final pH of EGTA ranged from pH 8 to 8.3. EGTA was stored at room temperature and was not kept for more

than one month. pH was measured using a calibrated Hanna Instrument HI8521 pH meter.

b) Pre-incubation of the effector CTLs with BFA, CMA and EGTA

CMA, BFA and EGTA were added to the CTLs at final concentrations of 10nM CMA, 40μM BFA and 3mM EGTA. CMA and BFA stock solutions were diluted 1:100 and 1:10 in 20% RPMI respectively and 1μl added to 99μl of CTL suspension in all wells to achieve the final concentrations shown above. 1μl of 300mM EGTA was added to CTLs in all wells. The 96-well plate was subsequently incubated for 2 to 3 hours at 37°C in 5% CO_2.

c) Target cells

Only autologous LCLs were used as targets in this cytotoxicity assay. These target cells were prepared in the same way as described in section 2.2.3.1 a) except that the number of cells used was $1x10^6$ to which 100μCi of ^{51}Cr were added. Following a 1 hour incubation with ^{51}Cr the LCLs were washed twice with RPMI or HBSS as previously described in 2.2.3.1 a) and finally resuspended in 10ml 20% RPMI. 100μl of these target cells were added to the CTLs as described below.

d) 4-hour incubation setup

The chromium-labelled autologous LCLs were added to the CTLs, pre-incubated with CMA, BFA and EGTA, as described in section 2.2.3.1.d). The remainder of the chromium release assay was performed as in section 2.2.3.1 d).

2.2.4 Recombinant vaccinia virus constructs

All work carried out using recombinant vaccinia virus constructs was done following appropriate training in the handling of genetically modified organisms. All materials that were in contact with recombinant vaccinia virus constructs were soaked in 1% w/v Virkon (VWR, Poole, UK) solution and/or treated with UV light to inactivate the virus.

2.2.4.1 Recombinant vaccinia virus constructs

Recombinant vaccinia virus constructs were obtained from Professor Rickinson, Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, UK. The recombinant vaccinia virus constructs express the following EBV-antigens: EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, LMP-1, LMP-2, LP and MA. A recombinant vaccinia virus construct not expressing an EBV antigen was included as a control in all experiments where these constructs were used.

2.2.4.2 TK-143B cells

TK-143B cells were purchased from the European Collection of Cell Culture (ECACC 91112502). TK-143B cells are adherent human bone osteosarcoma cells, which are thymidine kinase deficient but grow in DMEM with BrdU.

2.2.4.3 TK-143B cell bulk culture

Growing TK-143B cells were split by removing the 10% DMEM with BrdU cell culture medium, washing the monolayer with 10ml of 0.02% v/v versene, adding 5ml of trypsin (Invitrogen, Paisley, UK) and 5ml of 0.02% v/v versene and incubating for 3 to 5 minutes at 37°C in a 5% CO_2 atmosphere to detach the cells from the flask surface. Cells were decanted into a 50ml falcon tube and 20ml HBSS added to the flask to collect any remaining cells and added to the 50ml falcon tube. The cells were centrifuged at 300g for 7 minutes at 20°C and the supernatant was discarded. The cells were resuspended in 10ml 10% DMEM with BrdU and counted. Cells were seeded into fresh flasks – 5-6x10^6 cells for next day confluence and 2-3x10^6 cells for confluence in 2 to 3 days – and 50ml growth medium added.

2.2.4.4 TK-143B cell culture in 6-well plates

TK-143B cells were also seeded into 6-well plates for vaccinia virus construct titration. For confluence to be achieved the next day, cells were seeded at 3-5x10^5/well; for confluence to be achieved in 2-3 days 1x10^5 cells were seeded/well, and for confluence to be achieved in 4-5 days, 5x10^4 cells/well were seeded. Three

ml of growth medium were added to each well and the plate was incubated at 37°C in a 5% CO_2 atmosphere.

2.2.4.5 Recombinant vaccinia virus infection of TK-143B cells and harvest of recombinant vaccinia virus

10% DMEM with BrdU culture medium was removed from confluent monolayer of TK-143B cells. The cells were overlayed with the appropriate vaccinia virus construct ($2x10^7$pfu/3.5ml SPBS) and incubated at 37°C in a 5% CO_2 for 2 hours with gentle rocking every 30 minutes to ensure an even spread of virus over the cell monolayer. 45ml of 10% DMEM were added after the incubation and the cells were incubated for a further 3 to 4 days. After this incubation the cells began to round up and detach. Any remaining adherent cells were dislodged by gentle agitation and the cell suspension was poured into a 50ml falcon and centrifuged for 15 minutes at 800g. The supernatant was discarded. The cell pellet was resuspended in 1ml SPBS. The cell suspension was transferred to a 15ml falcon, ensuring that the tube was tightly sealed and covered with parafilm. The cells were freeze/thawed 3 times in liquid nitrogen/37°C waterbath and before each refreezing the cells were vortexed for 30 seconds. After freeze/thawing the cells were centrifuged for 5 minutes at 1200g. The supernatant was collected and transferred to a fresh 15ml falcon, aliquoted and stored at -80°C until required.

2.2.4.6 Recombinant vaccinia virus titration

900µl of SPBS was added to 7 wells of a 24-well tissue culture plate and 100µl of recombinant vaccinia virus was added to one well of the plate. 10-fold dilutions of the recombinant vaccinia virus were prepared in the remaining wells – 10^{-1} to 10^{-7}. 10% DMEM with BrdU culture medium was removed from a 6-well plate with confluent TK-143B cells and 400µl of recombinant vaccinia dilutions (10^{-5} to 10^{-7} prepared above) were added to the 6-well plate as shown in figure 2.2. A SPBS control was also included.

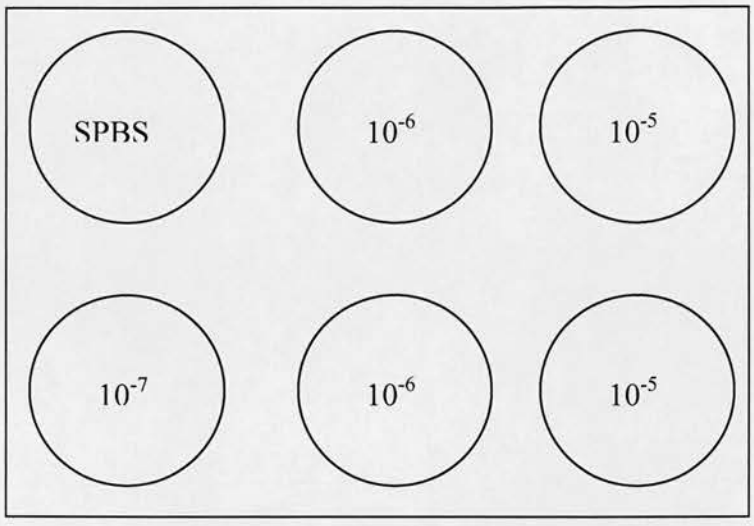


Figure 2.2: Titration of recombinant vaccinia virus.

The 6-well plate was incubated for 2 hours at 37°C in a 5% CO_2 atmosphere with gentle shaking every 30 minutes. 3ml of 10% DMEM were added to the wells thereafter and the plate was incubated for 3 days. 10% DMEM was removed and 2ml of 4% v/v normal buffered formaline (NBF) (made from 10% stock, BDH) was added and left for 30 minutes and then poured off. Enough 1% w/v Toludene blue (1g toludene blue, 10ml ethanol, 90ml dH_2O) was added to cover each well and left for 20 to 30 minutes. This was discarded and each well was washed with 2ml of PBS. The plate was left to dry and the plaques counted to calculate the recombinant vaccinia virus titre.

2.2.4.7 Vaccinia virus infection of dendritic cells

a) Monocyte isolation from PBMC

i) Magnetic labelling of non-monocytic cells

Dendritic cells were grown from monocytes isolated from fresh or frozen PBMC using a Monocyte Isolation Kit II (Milteyni Biotech). The isolation of monocytes was performed following the manufacturer's instructions. Briefly, PBMCs were

washed in 1-2 ml of MACS buffer (SPBS, 0.5% w/v BSA, 2mM EDTA) per 10^7 total cells and centrifuged at 4°C for 10 minutes at 300g. The supernatant was aspirated and the cell pellet was resuspended in 30µl MACS buffer per 10^7 total cells. 10µl of Fc receptor (FcR) blocking reagent and 10µl of biotin antibody cocktail (against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A) were added per 10^7 total cells, mixed well and incubated at 4°C for 15 minutes. 30µl of MACS buffer and 20µl of anti-biotin microbeads were added per 10^7 total cells, mixed well and incubated at 4°C for 15 minutes. Cells were washed by adding 10x labelling volume of MACS buffer and centrifuged at 4°C for 10 minutes at 300g. The supernatant was discarded and the cells were resuspended in 500µl MACS buffer.

ii) Magnetic cell separation

The MACS columns and MACS magnetic separator were chosen following the information given in table 2.2, which is adapted from the protocol supplied with the monocyte isolation kit.

Column	Max number of labelled cells	Max number of total cells	Separator
MS	10^7	$2x10^8$	MiniMACS
LS	10^8	$2x10^9$	MidiMACS

Table 2.2: Capacity of MACS columns and magnetic separators

The appropriate column was placed in the magnetic field of a suitable MACS separator dependant on cell number. The column was prepared by rinsing with the appropriate volume of MACS buffer: MS column – 500µl and LS column – 3ml. The cell suspension prepared above was applied to the column and the unlabelled monocyte-rich fraction was collected. The column was washed 3 times with MACS buffer: MS column – 3x500µl and LS column – 3x3ml. The washings were also collected and formed part of the unlabelled monocyte-rich fraction. The column was removed from the MACS separator and MACS buffer was added to the column –

MS column – 1ml and LS column – 5 ml. The magnetically labelled non-monocyte fraction was flushed out of the column using the plunger provided with the column. The purity of the labelled and unlabelled fraction was assessed by FACS staining as described in section 2.4.

b) Dendritic cell culture

The monocytes isolated above were resuspended at $1x10^6$ cells/ml in DC medium and interleukin (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF) were added to the monocytes at 40ng/ml (R&D Systems, Abingdon, UK). The cells were cultured for 6 days with the addition DC medium when required. The phenotype of the cells was established by FACS staining as described in section 2.4.

c) Infection of dendritic cells with recombinant vaccinia virus

10^4 dendritic cells generated above were infected with one recombinant vaccinia virus in 1 well of 96-well plate at a multiplicity of infection of 10:1 in 200µl of DC medium and incubated overnight at 37°C in a 5% CO_2 atmosphere. This was done for all 10 recombinant vaccinia virus constructs, including the control construct. 10^4 autologous LCL were also included in one well of the plate. The infected dendritic cells and the LCL were transferred to 15ml falcon tubes the next day and washed in 10ml HBSS or RPMI and centrifuged at 300g for 7 minutes at 20°C. The supernatant was discarded and the cell pellet loosened. The infected dendritic cells and LCL were ready to be used as target cells in the cytotoxicity assay described below.

2.2.4.8 4-hour chromium release cytotoxicity assay

a) Target cells

10 µCi of ^{51}Cr were added to each well of infected dendritic cells and to the LCLs and these were incubated for 1 hour at 37°C. These target cells were washed twice as described in section 2.2.3.1 a) and resuspended in a final volume of 300µl 20% RPMI.

b) Effector cytotoxic T-cells

Autologous CTLs were used as effector cells. $4x10^6$ CTL were resuspended in 4ml 20% RPMI and 100µl of CTL suspension was added to 36 wells of a 96-well U-bottomed plate. The assay was carried out at an E:T of 10:1.

c) Four-hour incubation step

100µl of each of the chromium-labelled target cells were added to 100µl CTL in triplicate wells. 100µl of chromium-labelled target cells were also added to 100µl 1% v/v Triton® X-100 in triplicate wells giving the maximum release chromium release by the target cells, and to 100µl 20% RPMI in triplicate giving the spontaneous release of chromium by the target cells. The effector and target cells were incubated for 4 hours at 37°C and the remainder of the assay was carried out as described in section 2.2.3.1 d).

2.2.5 Cell separations - CD4+ T-cell and CD56+ cell selection

CD4+ T-cells were positively selected from fresh and frozen PBMCs using three different cell separation methods – MACS (Miltenyi Biotech), Dynal Dynabeads with DETACHaBEAD (Dynal) and EasySep (StemCell Technologies). All three methods were used following the manufacturer's instructions and the purity of all cell fractions generated in all three methods was assessed by FACS staining as described in section 2.4. CD56+ cells were positively selected using EasySep.

2.2.5.1 MACS

CD4+ T-cell selection was performed following the manufacturer's instructions. The cells, buffer and MACS kit were kept at 4°C throughout the CD4+ T-cell selection.

a) Magnetic labelling of CD4+ T-cells

Briefly, PBMCs were washed in 1-2ml of MACS buffer per 10^7 total cells and centrifuged at 4°C for 10 minutes at 300g. The supernatant was aspirated and the cell pellet was resuspended in 80µl MACS buffer per 10^7 total cells. 20µl of CD4

MicroBeads were added per 10^7 total cells, mixed well and incubated at 4°C for 15 minutes. The cells were thereafter washed by adding 1-2ml of MACS buffer per 10^7cells and centrifuged at 4°C for 10 minutes at 300g. The supernatant was aspirated and the cell pellet was resuspended in 500µl of MACS buffer.

b) Magnetic separation of CD4+ T-cells

The magnetic separation of labelled CD4+ T-cells was carried out following the same protocol as described in section 2.2.4.7 a) ii), except that the fraction collected when the column was placed in the magnet was the unlabelled non-CD4+ T-cells and the fraction flushed out of the column once the column was removed from the magnet was the labelled CD4+ T-cells. The CD4+ T-cells were centrifuged out of MACS buffer for 7 minutes at 300g at 4°C. The supernatant was discarded and the cells resuspended in 10% RPMI and counted by trypan blue exclusion as described in section 2.2.2.2.

2.2.5.2 Dynal Dynabeads CD4 with DETACHaBEAD

CD4+ T-cell selection was performed following the manufacturer's instructions, except that FBS was used instead of FCS. The cells, buffer and Dynal Dynabeads CD4 and DETACHaBEAD kit components were all kept at 4°C throughout the CD4+ T-cell selection.

a) Dynabeads CD4 washing procedure

The Dynabeads were resuspended by vortexing and the required volume was transferred to a 5ml polystyrene round-bottom tube. 72µl of Dynabeads were required for 1ml of PBMC suspension. 1ml of SPBS/2% FBS buffer (SPBS, 2% v/v FBS) was added to the Dynabeads and mixed well. The 5ml polystyrene round-bottom tube containing the beads was placed in the Dynal MPC magnet for 30 seconds and the supernatant aspirated thereafter. The washed Dynabeads were resuspended in a volume of SPBS/2% FBS buffer equal to the volume required at the start of the washing procedure.

b) PBMC preparation

Fresh PBMC were resuspended at $0.5x10^7$ total cells/ml in SPBS/2% FBS buffer and were ready to use.

c) Positive isolation of CD4+ T-cells from PBMC

The PBMC prepared above were cooled to 2-8°C, added to the washed Dynabeads CD4 and incubated for 20 minutes at 2-8°C with both gentle tilting and rotating. The rosetted CD4+ T-cells were then isolated by placing the 5ml polystyrene round-bottom tube with the PBMC and the Dynabeads CD4 in the Dynal MPC magnet for 2-3 minutes. The supernatant was aspirated and the rosetted CD4+ T-cells remained attached to the side of the test tube by the Dynal MPC magnet. The rosetted CD4+ T-cells were washed 4-5 times in SPBS/2% FBS buffer. Finally, the rosetted CD4+ T-cells were resuspended in 100µl RPMI/1%FBS solution (RPMI, 1% v/v FBS). These CD4+ T-cells were bound to the Dynabeads CD4.

d) Detachment of Dynabeads CD4 from CD4+ T-cells

10µl of DETACHaBEAD were added to every 100µl of rosetted CD4+ T-cells and incubated for 45 to 60 minutes at room temperature with gentle mixing. The tube was subsequently placed in the Dynal MPC magnet for 2 minutes. This released the CD4+ T-cells into the supernatant. These were transferred to a fresh test tube. The beads remaining in the tube placed in the magnet were washed 2-3 times with 500µl RPMI/1%FBS and the supernatant was collected. Finally, the detached CD4+ T-cells were washed in 10ml RPMI/1%FBS and centrifuged for 7 minutes at 200g to remove any remaining DETACHaBEAD. The supernatant was aspirated and the CD4+ T-cells resuspended in 10% RPMI and counted by trypan blue exclusion as described in section 2.2.2.2.

2.2.5.3 EasySep

CD4+ T-cells were positively selected from fresh or frozen PBMC and CD56+ cells from frozen CTLs. The positive selection for both CD4+ T-cells and CD56+ cells was performed following manufacturer's instructions.

a) Cell suspension preparation (PBMCs and CTLs)

Cells were resuspended in EasySep buffer (SPBS, 2% v/v FBS, 1mM EDTA) at a concentration of $1x10^8$ cells/ml. Where 10^7 or less cells were used, these were resuspended in 100µl EasySep buffer. The PBMC suspension was made up in a 5ml polystyrene round-bottom tube.

b) CD4+ T-cell and CD56+ cell selection

CD4+ T-cell or CD56+ cell EasySep positive selection cocktail (contains a combination antibodies bound to bispecific tetrameric antibody complexes which are directed against CD4 or CD56 and dextran) was added to the PBMC suspension at 100µl/ml of cell suspension. The positive selection cocktail and the PBMC were well mixed and incubated at room temperature for 15 minutes. The EasySep magnetic nanoparticles were vortexed to be in a uniform suspension and 50µl of nanoparticles were added to 1ml of cell suspension. The cells and nanoparticles (magnetic dextran iron particles in water) were mixed and incubated for 10 minutes at room temperature. The volume of the cell suspension was brought up to 2.5ml using EasySep buffer and mixed gently. The tube was placed in the magnet for 5 minutes. The supernatant was thereafter poured out in one continuous movement. The tube was removed from the magnet, 2.5ml EasySep buffer added to the tube and the cells mixed gently. The tube was placed back in the magnet for 5 minutes. The final 2 steps where EasySep buffer was added to the tube and placed in the magnet were repeated once more. Finally the cells remaining in the tube, after pouring out the last 2.5ml of EasySep buffer, were the selected cells. These were resuspended in 10% RPMI and counted by trypan blue exclusion as described in section 2.2.2.2.

2.3 Molecular methods

2.3.1 Consumables, kits, reagents and equipment used

2.3.1.1 Molecular methods' consumables

96-well U-bottomed plates were supplied by Nunc and all other plasticware was supplied by Greiner-Bione, SLS and Sterilin. Thin-walled 0.2ml PCR tubes and 24-well PCR plates were supplied by Anachem.

2.3.1.2 Kits, reagents and PCR primers

Qiashredder spin columns and RNeasy Mini Kit were both purchased from Qiagen. β-mercaptoethanol and multi-purpose agarose were supplied by Sigma. Biochemical grade ethanol and nuclease-free water were supplied by BDH. Primers were obtained from Sigma-Genosys. ThermoScript™ Reverse Transcriptase cDNA synthesis kits and 100 base pair (bp) ladder were both purchased from Invitrogen and the RQ1 RNase-Free DNase kits were purchased from Promega. Ethidium bromide was supplied by Fisher Scientific. All components required for T-cell receptor spectratyping PCR reactions were AmpliTaq Gold® reagents purchased from Applied Biosystems and T-cell receptor spectratyping size standard GeneScan™-500 LIZ™ and Hi-Di™ formamide were also purchased from Applied Biosystems.

2.3.1.3 Equipment used

All centrifugation steps were carried out using an MSE Microfuge (Sanyo). Reverse-transcriptase PCR (RT-PCR) reactions were carried using a T3 Thermalcycler (Biometra) and Robocycler 96 (Stratagene). T-cell receptor spectratyping was carried out on an ABI Sequencer 3730 (Applied Biosystems) at the School of Biological Sciences Sequencing Services, The University of Edinburgh. Spectratyping analysis was performed using GeneMapper Software v3.7 from Applied Biosystems.

2.3.2 RNA / cDNA methods

2.3.2.1 RNA extraction

All cells from which total ribonucleic acid (RNA) was to be extracted were washed in SPBS twice before being pelleted and either used immediately or stored at -20°C until required. Total RNA was extracted using an RNeasy Mini Kit and Qiashredder spin columns. All buffers mentioned below were included in the RNeasy Mini Kit. Cell pellets were loosened and resuspended in proprietary RLT buffer (which contains guanidine thiocyanate that has a strong but temporary effect on protein denaturation) supplemented with β-mercaptoethanol (which is a reducing agent that irreversibly denatures RNases by reducing disulfide bonds and destroying native conformation of the enzymes) (10µl β-mercaptoethanol/ml of RLT buffer) – 350µl for $<5x10^6$ cells and 600µl for $5\text{-}10x10^6$ cells. The RLT buffer mixed with β-mercaptoethanol was stored at room temperature and kept for no more than 2 weeks. This cell suspension was added onto a Qiashredder spin column and centrifuged at 8000g for 2 minutes to generate a cell lysate. An equal volume of 70% v/v ethanol, made using nuclease-free water, was added to the cell lysate. A maximum of 700µl of lysate was applied to RNeasy mini spin column and centrifuged for 15 seconds at 8000g and the flow through discarded. If the volume exceeded 700µl this step was repeated, using the same RNA mini spin column. The flow through was again discarded. 700µl of RW1 buffer were added to the column and this was centrifuged for 15 seconds at 8000g and the flow through discarded. The RNeasy mini spin column was thereafter transferred to a clean 2ml collection tube. 500µl of proprietary RPE buffer (which also contains guanidine thiocyanate) were added to the column and this was centrifuged again for 15 seconds at 8000g. RPE buffer was made by adding 45ml of 100% technical grade ethanol to 11ml of stock RPE provided by the manufacturer. Another 500µl of RPE buffer were added to the column and centrifuged for 2 minutes at 8000g. The flow through was discarded and the centrifugation step repeated for 1 minute. The RNeasy mini spin column was transferred to a clean 1.5ml eppendorf tube. Finally, the RNA was eluted from the RNeasy mini spin column by adding 30µl to 50µl of RNase-free water to the column

and centrifuging this for 1 minute at 8000g. The concentration of eluted RNA was measured using a spectrophotometer as described below.

2.3.2.2 Measurement of RNA concentration

RNA concentration was determined using the following formula:

RNA concentration = Absorption at 260nm (A^{260}) x 40 x dilution factor where an A^{260} of 1.0 is equivalent to 40mg/ml of RNA using a GeneQuant UV spectrophotomer (Pharmacia Biotech, Little Chalfont, UK). The spectrophotometer was blanked at A^{260} using 4µl of RNase-free water from the RNeasy Mini kit that was used to elute the RNA. 4µl of eluted RNA were added to a quartz capillary (Amersham) to determine the RNA concentration, ratio and absorbance. Pure RNA has an A^{260}/A^{280} ratio between 1.8 and 2. A value above 2 indicated protein contamination and a value below 1.8 indicated poor RNA quality (Sambrook and Russell, 2001).

2.3.2.3 DNase treatment of RNA

All RNA samples were systematically treated using an RQ1 RNase-Free DNase kit to remove any contaminating DNA. DNase digestion was carried out following the manufacturer's instructions as outlined in table 2.3.

Kit component	Volume	Component composition
RNA in water	1-8µl	-
RQ1 RNase-Free DNase 10x Reaction Buffer	1µl	400mM Tris-HCl (pH 8.0), 100mM $MgSO_4$ and 10mM $CaCl_2$
RQ1 RNase-Free DNase (from bovine pancreas)	1U/µg RNA	RQ1 DNase supplied in 10mM HEPES (pH 7.5), 50% v/v glycerol, 10mM $CaCl_2$ and 10mM $MgCl_2$.
Nuclease-free water to a final volume of	10µl	

Table 2.3: RQ1 DNase treatment of RNA.

The RNA and RQ1 components were incubated at 37°C for 30 minutes. The above reaction was terminated by adding 1µl of RQ1 stop solution followed by a 10 minute incubation at 65°C. This inactivated the DNase. RNA was thereafter stored at -80°C until required.

2.3.2.4 Complementary DNA synthesis

A ThermoScript™ Reverse Transcriptase kit was used for the synthesis of first-strand complementary DNA (cDNA) from the RNA generated above. This was performed following the manufacturer's instructions. Briefly, components of the kit were added to the RNA (between 50ng and 5µg RNA) as indicated in table 2.4. Only DNase-treated RNA was used.

Kit component	Volume	Component composition
50ng/µl random hexamers	1µl	N/A
10mM dNTP mix	2µl	N/A
10pg to 5µg RNA	x µl	-
DEPC-treated water	To a final volume of 12µl	-

Table 2.4: Components of ThermoScript Reverse Transcriptase kit added to RNA.

This mixture was incubated for 5 minutes at 65°C to denature the RNA. The components listed in table 2.5 were added thereafter to the denatured RNA:

Kit components	Volume	Component composition
5x cDNA synthesis buffer	4µl	250mM tris acetate (pH 8.4 at room temperature), 375mM potassium acetate, 40mM magnesium acetate
0.1M DTT	1µl	0.1M dithiothreitol
RNaseOUT™ (40U/µl)	1µl	Recombinant ribonuclease inhibitor
DEPC-treated water	1µl	-
ThermoScript™ RT (15U/µl)	1µl	Avian reverse transcriptase

Table 2.5: Components of ThermoScript Reverse Transcriptase kit added to denatured RNA.

cDNA was finally synthesised when the components in table 2.5 were added to the denatured RNA and incubated following at 25°C for 10 minutes, 50°C for 60 minutes and 85°C for 5 minutes. The cDNA generated was stored at -20°C until required.

2.3.3 Reverse Transcriptase-Polymerase Chain Reaction methods

2.3.3.1 Reverse transcriptase-polymerase chain reaction master-mix

A master-mix was prepared for all RT-PCR reactions described below. The reagents of this master-mix, their composition and their supplier are given in table 2.6.

Reagents	Composition/Concentration	Suppliers
10x PCR buffer	10mM Tris-HCl (pH 9.0 at 25°C), 50mM KCl and 0.1% Triton® X-100	Promega
25mM Mg^{2+}	25mM $MgCl_2$	Promega
dNTPs	dATP, dCTP, dGTP, dTTP (20mM of each dNTP)	Amersham
Forward primers	100µM	Sigma-Genosys
Reverse primers	100µM	Sigma-Genosys
Nuclease-free water	H_2O	BDH
Taq DNA polymerase from *Thermus aquaticus* strain YT 1	DNA polymerase	Promega

Table 2.6: RT-PCR master-mix components, compositions and suppliers.

All primers used were at a concentration of 100µM. All PCR products were visualised by agarose gel electrophoresis as described in section 2.3.3.4. All PCR products were stored at -20°C.

2.3.3.2 β-actin RT-PCR

β-actin PCR was performed on all cDNA samples to check the quality of the cDNA synthesised. β-actin PCR was also performed on all RNA used to generate cDNA to check for any DNA contamination of the RNA. The primer sequences used are given in table 2.7 below:

Primer name	5' to 3' sequence	Product size (bp)
β-actin forward	CTC CTT AAT GTC ACG CAC GAT TTC	540
β-actin reverse	CGC CCC CGC CCC AGG CAC CA	

Table 2.7: β-actin primer sequences.

The composition of the master-mix for β-actin RT-PCR is given in table 2.8.

Reagent	Volume in µl
10x PCR buffer	2.5
25mM Mg^{2+}	1.5
dNTPs	0.25
β-actin forward primer	0.2
β-actin reverse primer	0.2
Nuclease-free water	18.23
Taq DNA polymerase	0.125
cDNA or RNA	2

Table 2.8: β-actin RT-PCR reaction master-mix.

The RT-PCR was performed using the T3 Thermalcycler under the conditions given in table 2.9. A positive control for β-actin was included in each RT-PCR run. A negative control consisting of 2µl of NFH_2O instead of cDNA or RNA was included in each RT-PCR run.

Number of cycles	Temperature (°C)	Time (minutes)
1	95°C	5
35	95°C	1
	65°C	2
1	72°C	10
1	4°C	hold

Table 2.9: β-actin RT-PCR cycling conditions.

2.3.3.3 FasL, Granulysin, Granzyme B and Perforin RT-PCR

The sequences of the primers used are given in table 2.10. These were designed using the Primer3 software available at:
http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.

Primer name	5' to 3' sequence	Expected product size (bp)
FasL forward	TGGCCTTGTGATCAATGAAA	155
FasL reverse	TCATCATCTTCCCCTCCATC	
Granulysin forward	CCAGGTCTGGTCTTCTCTCG	202
Granulysin reverse	TGGGCTTATCCACCATCTTC	
Perforin forward	ATTCACCCTGTCCAAACTGC	234
Perforin reverse	GTGGGCAAAGAAGACAGAC	
Granzyme B forward	GGAGGCCCTCTTGTGTGTAA	167
Granzyme B reverse	ATTACAGCGGGGGCTTAGTT	

Table 2.10: FasL, granulysin, perforin and granzyme B primer sequences.

The master-mix for each RT-PCR reaction is given in the table below:

Reagent	Perforin	Granzyme B	Granulysin	FasL
10x PCR buffer	2.5µl	2.5µl	2.5µl	2.5µl
25mM Mg^{2+}	1.5µl	1.5µl	1.5µl	1.5µl
dNTPs	0.25 µl	0.25µl	0.25µl	0.25µl
Forward primer (50pM)	0.20µl	0.20µl	0.20µl	0.20µl
Reverse primer (50pM)	0.20µl	0.20µl	0.20µl	0.20µl
Nuclease-free water	17.65µl	17.65µl	17.65µl	17.65µl
Taq DNA polymerase	0.125µl	0.125µl	0.125µl	0.125µl
cDNA	2.5µl	2.5µl	2.5µl	2.5µl

Table 2.11: FasL, granulysin, perforin and granzyme B RT-PCR master-mix. The minimum volume of cDNA used was 1µl, and although the volume of cDNA was increased in some cases, this volume never exceeded 2.5µl. Where the volume of cDNA was increased, the volume of NFH_2O was decreased accordingly to maintain a total reaction mix volume of 25µl.

The RT-PCR cycling conditions (duration and temperature of the melting, annealing and elongation phases as well as the number of cycles) were established based on the data provided with the primers (melting temperature and expected RT-PCR product sizes). As the melting temperatures of the primers and the expected RT-PCR product sizes were similar for all four cytotoxic molecules the same conditions were used. The RT-PCR cycling conditions for FasL, granulysin, perforin and granzyme B were all the same and are given in table 2.12.

RT-PCR cycling conditions	
94°C – 5 min	1x
95°C – 30 s 57°C – 30 s 72°C – 45 s	35x
72°C – 8min	1x
4°C – hold	

Table 2.12: FasL, granulysin, perforin and granzyme B RT-PCR cycling conditions.

2.3.3.4 Agarose gel electrophoresis of RT-PCR products

2% agarose was prepared using multi-purpose agarose and 0.5% TBE buffer (45mM Tris-borate, 1mM EDTA in 20 litres (l) dH_2O). The solution was heated in a microwave with regular swirling until the agarose had dissolved. The agarose was allowed to cool and 1µl of ethidium bromide (10mg/ml) (Fisher Scientific) was added to every 100ml of agarose. The molten agarose was poured into the appropriate cast with comb and allowed to set.

The solidified gel was submerged with 0.5% TBE. 10µl of RT-PCR products were mixed with 2µl of PCR loading buffer 10x (1ml 0.5M EDTA pH 8.0, 3g sucrose, 500µl 1% bromophenol blue, 500µl 1% xylene cyanol, 3ml dH_2O) and loaded into the wells of the gel. 10µl of 100 base pair ladder (Invitrogen) was also loaded into every gel. The electrophoresis was run until the 100 base pair ladder had separated appropriately along with the RT-PCR products.

The 100 bp ladder and RT-PCR products were visualised in the gel using a UVP Transilluminator (Ultra-Violet Products, Cambridge, UK). Gel images were printed on Sony heat-sensitive paper supplied by SLS.

2.3.4 T-cell receptor Vβ spectratyping

T-cell receptor (TCR) repertoire diversity was characterised by TCR Vβ spectratyping by RT-PCR. This was done following a method previously described by Foster *et al* (2004), which measures the differing length of the CDR 3 of the TCR β chain.

2.3.4.1 Primer sequences

The primer sequences used in TCR spectratyping are given in table 2.13. These consist of 23 forward primer for Vβ sequences and 1 reverse primer for constant chain Cβ conjugated to the fluorescent dye carboxyfluorescein (FAM) (Foster *et al*, 2004).

TCR Vβ	Sequence	Expected product (bp)
1	GCACAACAGTTCCCTGACTTGCAC	195-207
2	TCATCAACCATGCAAGCCTGACCT	195-207
3	GTCTCTAGAGAGAAGAAGGAGCGC	190-208
4	ACGATCCAGTGTCAAGTCGAT	334-346
5	CTGATCAAAACGAGAGGACAGCA	354-375
6	TCAGGTGTGATCCAATTTC	329-347
7	CCTGAATGCCCCAACAGCTCTC	190-214
8	GGTGACAGAGATGGGACAAGA	355-373
9	CACCTAAATCTCCAGACAAAGCT	194-212
11	TGTTCTCAAACCATGGGCCATGAC	321-333
12	GrCATGGGCTGAGGCTGAT	267-290
13	CTCTCCTGTGGGCArGTC	408-425
14	ACCCAAGTACCTCATCACAG	328-383
15	AGTGTCTCTCGACAGGCACAG	193-208
16	AAAGAGTCTAAACAGGATGAGCC	241-256
17	TTTCAGAAAGGAGATATAGCT	226-241
18	AGCCCAATGAAAGGACACAGTCAT	325-337
20	CTCTGAGGTGCCCCAGAA	218-227
21	GGCTCAAAGGAGTAGACTCC	185-200
22	ATGAAATCTCAGAGAAGTCT	234-252
23	GATCAAAGAAAAGAGGGAAAC	358-370
24	TACCCAGTTTGGAAAGC	353-368
25	CAGGTATGCCCAAGGAAAGA	226-241
Cβ(FAM)	TTCTGATGGCTCAAACAC	

Table 2.13: 23 Vβ forward primer sequences and 1 reverse constant chain (Cβ) primer conjugated to FAM.

All the primers were diluted to 100μM stock solutions. Primers Vβ 1-25 were diluted further to 4μM. 5μl of each forward primer was aliquoted in 23 wells of a 0.2ml 24-well PCR plate.

2.3.4.2 Reaction master-mix

The TCR RT-PCR reaction master-mix is given in table 2.14.

Reagent	Volume in μl	Final concentration
10 x PCR Buffer	2	1 x
25mM Magnesium	1.6	2mM
20mM dNTP's	0.2	200μM
100μM 3'primer (Cβ-FAM)	0.2	1μM
Amplitaq gold (5U/μl)	0.1	0.5U
NFH_2O	9.9	N/A
cDNA	1	N/A

Table 2.14: TCR RT-PCR reaction master-mix. N/A: not applicable.

The volume of each reagent was multiplied by the number of primers being amplified. A new reaction master-mix was prepared for each cDNA sample to be tested. cDNA used in any master-mix was synthesised using 1μg of RNA (as described in section 2.3.2.1). This RNA was DNase treated and the quality of the cDNA synthesised was checked by β-actin RT-PCR (as described in sections 2.3.2.3 and 2.3.3.2). The master-mix was vortexed, pulse centrifuged briefly and 15μl of master-mix was added to each well of a 24-well PCR plate containing 5μl of forward primer.

2.3.4.3 T-cell receptor RT-PCR cycling conditions

The RT-PCR cycling conditions used are detailed in table 2.15.

Number of cycles	Temperature (°C)	Time
1	95°C	5 minutes
	95°C	30 seconds
3	58°C	30 seconds
	72°C	45 seconds
1	72°C	5 minutes
1	4°C	hold

Table 2.15: TCR RT-PCR cycling conditions.

10µl of TCR RT-PCR products were visualised as described in section 2.3.3.4.

2.3.4.4 T-cell receptor RT-PCR product preparation

A 1:10 dilution of the TCR RT-PCR products generated above was prepared in 96-well U-bottomed plate using NFH_2O. The size standard GeneScan™-500 LIZ™ and Hi-Di™ formamide mix was prepared by adding 2µl of size standard to 1ml of formamide. 9µl of formamide/standard mix was added to each well of a 96-well plate specifically designed for use with the ABI 3730 sequencer. 1µl of 1:10 diluted TCR RT-PCR product was added to the 9µl of formamide/standard mix. The plate was then sealed and analysed in the ABI 3730 sequencer.

2.3.4.5 Data analysis

All data generated by the ABI 3730 sequencer was analysed using GeneMapper software v3.7.

2.4 Fluorescence associated cell sorting

2.4.1 Antibodies and controls

Details of all the antibodies used are given in table 2.16.

Antibody	Clone	Isotype	Conjugated fluorochrome	Volume used/ 10^6 cells	Manufacturer
Extracellular markers					
CD3	UCHT-1	IgG_1, κ	FITC, PE, PE Cy5	20µl/10^6 cells	BD Pharmingen
CD4	RPA-T4	IgG_1, κ	APC, FITC, PE, PE Cy5	20µl/10^6 cells	BD Pharmingen
CD8	RPA-T8	IgG_1, κ	APC, FITC, PE, PE Cy5	20µl/10^6 cells	BD Pharmingen
CD11c	B-Ly6	IgG_1, κ	PE	20µl/10^6 cells	BD Pharmingen
CD14	rmC5-3	IgG_1, κ	PE	20µl/10^6 cells	BD Pharmingen
CD19	HiB19	IgG_1, κ	FITC, PE	20µl/10^6 cells	BD Pharmingen
CD27	M-T271	IgG_1, κ	FITC	20µl/10^6 cells	BD Pharmingen
CD28	CD28.2	IgG_1, κ	FITC	20µl/10^6 cells	BD Pharmingen
CD45	HI30	IgG_1, κ	FITC	20µl/10^6 cells	BD Pharmingen
CD45RA	HI100	IgG_{2b}, κ	FITC	20µl/10^6 cells	BD Pharmingen
CD45RO	UCHL-1	IgG_{2a}, κ	FITC	20µl/10^6 cells	BD Pharmingen
CD56	B159	IgG_1, κ	FITC, PE	20µl/10^6 cells	BD Pharmingen
CD62L	Dreg-56	IgG_1, κ	FITC	20µl/10^6 cells	BD Pharmingen
CD69	FN50	IgG_1, κ	FITC	20µl/10^6 cells	BD Pharmingen
DC-SIGN	NA	IgG_{2b}, κ	FITC	20µl/10^6 cells	R&D Systems
Intracellular markers					
Perforin	δG9	IgG_{2b}, κ	PE	20µl/10^6 cells	BD Pharmingen
Granzyme B	GB12	IgG_1	PE	10µl/10^6 cells	Caltag Medsystems
FasL	Alf-2.1	IgG_{2a}	PE	10µl/10^6 cells	Caltag Medsystems

Table 2.16: Antibodies used in extra- and intra-cellular FACS staining. APC: allophycocyanin; FITC: fluorescent isothiocyanin; PE: phycoerythrin; PE Cy5: phycoerythrin cychrome 5. NA: not available.

Corresponding isotype controls were used for all of antibodies shown in table 2.16 and detailed in table 2.17.

Antibody isotype	Clone	Conjugated fluorochrome	Concentration	Manufacturer
IgG_1, κ	MOPC-21	APC, FITC, PE, PE Cy5	20µl/10^6 cells	BD Pharmingen
IgG_{2a}, κ	G11-178	FITC	20µl/10^6 cells	BD Pharmingen
IgG_{2b}, κ	27-35	FITC, PE	20µl/10^6 cells	BD Pharmingen
IgG_1	NA	PE	10µl/10^6 cells	Caltag Medsystems
IgG_{2a}	NA	PE	10µl/10^6 cells	Caltag Medsystems

Table 2.17: Isotype control antibodies. NA: not available.

2.4.2 Solutions and buffer used

Fluorescence associated cell sorting (FACS) buffer was made up using SPBS, 1% w/v bovine serum albumin (BSA), 5mM EDTA, 0.1% w/v sodium azide. Both BSA and EDTA were supplied by Sigma and sodium azide by BDH. BD FACS™ Permeabilizing Solution 2 10x concentrate (BD Biosciences) and CellFIX™ 10x concentrate (BD Biosciences) were both diluted 1:10 with dH_20 before use. 1:10 diluted CellFIX™ was kept for up to 1 month at room temperature.

2.4.3 Equipment used

All centrifugation steps were carried out using a Sanyo MSE Mistral 3000E (Rotor 43124-129). FACS was performed using either a FACScan or a FACSCalibur in conjunction with the CellQuest Software (Becton Dickinson). Acquired events were analysed using the CellQuest Software.

2.4.4 Extracellular marker staining

50µl of FACS buffer were added to 5ml round-bottomed tubes. The appropriate volume of extracellular marker antibody was added to the appropriate tubes. An unstained control was included in every experiment where no antibody was added to the buffer. 100µl of cell suspension, at 1 x 10^6 cells/ml in FACS buffer, was added to each tube, mixed gently and incubated for 20 minutes at 4°C in the dark. The cells were washed twice in 1ml of FACS buffer and centrifuged at 200g for 5 minutes. The supernatant was discarded and the cell pellet loosened. The cells were fixed in 200µl of 1:10 diluted CellFIX™ and incubated on rotating shaker for 10 minutes at room temperature. 300µl of FACS buffer were finally added to the cells, which were stored at 4°C in the dark until flow cytometry acquisition and analysis.

2.4.5 Intracellular staining

The cells were first stained for extracellular markers, as described in section 2.4.4 above, except that the cells were not incubated with CellFIX™ after the second wash but were instead incubated with 500µl BD FACS™ Permeabilizing Solution 2 diluted 1:10 for 10 minutes at room temperature. The cells were washed twice with 1ml FACS buffer and centrifuged at 200g for 5 minutes. The supernatant was discarded and the appropriate volume of intracellular antibody was added to the cells, mixed gently and incubated for 30 minutes at 4°C in the dark. Thereafter, the cells were washed twice with 1ml FACS buffer and centrifuged at 200g for 5 minutes and fixed as described in section 2.4.4.

2.4.6 Acquisition and analysis of events

Between 1000 and 10000 events, depending on the number of stained cells available, were acquired on either the FACScan or the FACSCalibur. These acquired events were subsequently analysed using the CellQuest software.

2.5 Western blotting for granulysin

2.5.1 Consumables and equipment used for western blot

CellLytic™-M mammalian cell lysis buffer and protease inhibitor cocktail for mammalian cells were both purchased from Sigma. Pre-cast NuPAGE® Novex High Performance 12% Bis-Tris Gel were purchased from Invitrogen, as well as all other reagents used, except the Rainbow Ladder size standard, which was purchased from Amersham Biosciences. Methanol was supplied by BDH. Hybond ECL nitrocellulose membranes were purchased from Amersham Biosciences. Whatman filter paper was supplied by VWR. Blocking solution was made using PBS (Oxoid, Basingstoke, UK) (1 tablet in 100ml dH_2O (NaCl 8g/l, KCl 0.2g/l, Na_2HPO_4 1.15g/l, KH_2PO_4 0.2g/l, pH7.2) 2% w/v BSA, 0.01% v/v Triton®-X). NBT/BCIP ready-to-use tablets were bought from Roche (Lewes, UK).

2.5.2 Equipment used for western blot

The cell lysis step was carried out using a Blood Tube Rotator SB1 (Stuart Scientific, Stone, UK). The centrifugation step was carried out using a temperature-controlled SciQuip1-15K centrifuge (Sigma – rotor ID 12124). Running and blotting of western blot was done using an *X*cell *SureLock*™ Mini-Cell from Invitrogen. Antibody incubations were carried out on a Roller Mixer SR T (Stuart Scientific).

2.5.3 Antibodies

The details of the antibodies used in western blot are detailed in table 2.18.

Antibody	Clone	Isotype	Concentration	Supplier
Anti-granulysin - 1° antibody	DH4	Mouse anti-human IgG_1,κ	1.64mg/ml	Gift from Prof C Clayberger[1]
Goat anti-mouse IgG (Fab specific) alkaline phosphatase conjugated - 2° antibody	NA	Goat anti-mouse IgG_1,κ alkaline phosphatase conjuagated	NA	Sigma

Table 2.18: Antibodies used in western blotting for granulysin. [1]Professor Carol Clayberger, Dept Pediatrics, Stanford University, Stanford, California, USA. mg: milligram. NA: not available.

2.5.4 Cell lysis

All cells used for western blotting were washed with SPBS before being stored at -20°C as dry cell pellets. These cell pellets were allowed to thaw before being lysed. 125µl of cell lysis buffer and 1.25µl protease inhibitor cocktail were added to the loosened cell pellet and this was incubated, with continuous rotation, using the blood tube rotator at 4°C for 25 minutes. The cell lysate was then centrifuged at 15000g for 10 minutes at 4°C. The protein supernatant was stored at -80°C in 15µl aliquots until required or used immediately and the pellet discarded.

2.5.5 Protein sample preparation and gel running

5µl of NuPAGE® LDS sample buffer (4x) and 2µl of NuPAGE® reducing agent (10x) were added to 13µl of protein sample and heated to 70°C for 10 minutes. 1x SDS running buffer was prepared by adding 50ml 20x NuPAGE® MES SDS running buffer to 950ml dH_2O. The upper buffer chamber of the *X*cell *SureLock*™

Mini-Cell was filled with 200ml 1x SDS running buffer and 500µl NuPAGE® antioxidant. The lower chamber was filled with 600ml SDS running buffer. The entire prepared sample was loaded in the gel. 10µl of Rainbow Ladder was loaded into every gel, as well as a positive control. The positive control for granulysin was a CD56+ rich T-cell line. The gel was run for 35 minutes at 200V constant.

2.5.6 Western transfer protocol

1l of 1x NuPAGE® transfer buffer was prepared by adding 50ml of 20x NuPAGE® transfer buffer, 100ml methanol and 1ml NuPAGE® antioxidant to 850ml dH_2O. Soaking pads, Hybond ECL nitrocellulose membrane and Whatman filter paper were soaked in 700ml of 1x NuPAGE® transfer buffer. A sandwich comprising 2 soaking pads, 2 Whatman filter paper squares, the gel, the Hybond ECL nitrocellulose membrane, 2 further filter paper squares and 2 further soaking pads was made and placed into the cathode core of the blot module. The blot module was finally assembled and fitted in the *X*cell *SureLock*™ Mini-Cell. Enough 1x NuPAGE® transfer buffer was added to the blot module until the soaking pads/Whatman filter paper/gel/Hybond ECL nitrocellulose membrane sandwich was covered. The lower buffer chamber was filled with 650ml dH_2O. The gel was transferred using 30V constant for 1 hour.

2.5.7 Primary and secondary antibody incubations

The sandwich of soaking pads/Whatman filter paper/gel/Hybond ECL nitrocellulose membrane was disassembled and the Hybond ECL nitrocellulose membrane was blocked for 30 minutes at room temperature with blocking solution on the roller mixer. The membrane was thereafter washed 3 times in PBS for 1 minute. 5ml of blocking solution and 5µl of anti-granulysin antibody (1:1000 dilution) were added to the membrane and incubated for 30 minutes at room temperature and then overnight at 4°C on the roller mixer. The membrane was washed 3 times in PBS for

1 minute. 5ml of blocking solution and 5µl of goat anti-mouse IgG (Fab specific) alkaline phosphatase conjugated antibody (1:1000 dilution) were added to the membrane and incubated for at least 2 hours at room temperature on the roller mixer. The membrane was washed 3 times in PBS for 1 minute.

2.5.8 Development Hybond ECL nitrocellulose membrane

The membrane was developed using an NBT/BCIP tablet dissolved in 10ml dH_2O. The membrane was developed in the dark for 3 to 5 minutes or until bands reached the desired intensity and washed in dH_2O to stop the reaction and allowed to dry.

2.6 Statistical analysis

The Mann-Whitney test was used to analyse non-parametric data. This was carried out using the software Prism 4.0 for Macintosh (GraphPad software, Inc., San Diego, USA).

Chapter 3

Growth and phenotype of CTLs

3.1 Introduction

3.2 *Ex vivo* generation and phenotype of CTLs

3.3 Investigation into CD4+ T-cells and CD8+ T-cells interactions

3.4 Discussion

3.1 Introduction

Ex vivo generated EBV-specific CTLs can take up to three months to produce. They have already been used to treat PTLD successfully in iatrogenically immunosuppressed transplant patients (Haque *et al*, 2001; Haque *et al*, 2002), however little is known about their development during the production period. Therefore, the growth and phenotype of CTLs was investigated in order to establish how the CTLs evolved in response to weekly autologous LCL stimulations.

3.2 *Ex vivo* generation and phenotype of CTLs

3.2.1 Growth of CTLs

The CTLs were generated using peripheral blood mononuclear cells (PBMCs) from 12 healthy EBV positive blood donors enrolled in a phase II clinical trial funded by Cancer Research UK. The HLA-type of each donor is given in table 3.1.

Donor number	HLA type
3	A2,68(28); B50(21),60(40); DR17(3),4
12	A3,24; B7; DR11(5),13(6)
16	A26,32; B55(22),64(14); DR7,14(6)
28	A1,2; B39(16),18; DR17(3),7
31	A1,2; B7,57(17); DR7,9
55	A2,68(28); B51(5),62(15); DR4,(13)6
66	A3,26; B7,41; DR15(2)
68	A2; B7; DR15(2)
75	A3; B35, DR1,13(6)
81	A3,30; B7,57(17); DR15(2),7

Table 3.1: HLA type of PBMC donors.

14 CTLs were generated using PBMCs from above mentioned donors. As a proportion of the CTLs were cryopreserved at particular weekly stimulations (S), the absolute CTL growth number could not be determined but, as at every weekly stimulation the density of the CTLs was adjusted to $1x10^6$ CTL/ml, this value was taken as a "benchmark" to follow any proportional increase or decrease in CTL numbers. The average proportional growth of the CTLs generated is shown in figure 3.1 as red bars. The $1x10^6$ CTL/ml "benchmark" is shown in figure 3.1 as a bold line. The average viability of the CTLs was also determined and this represented by the blue line in figure 3.1.

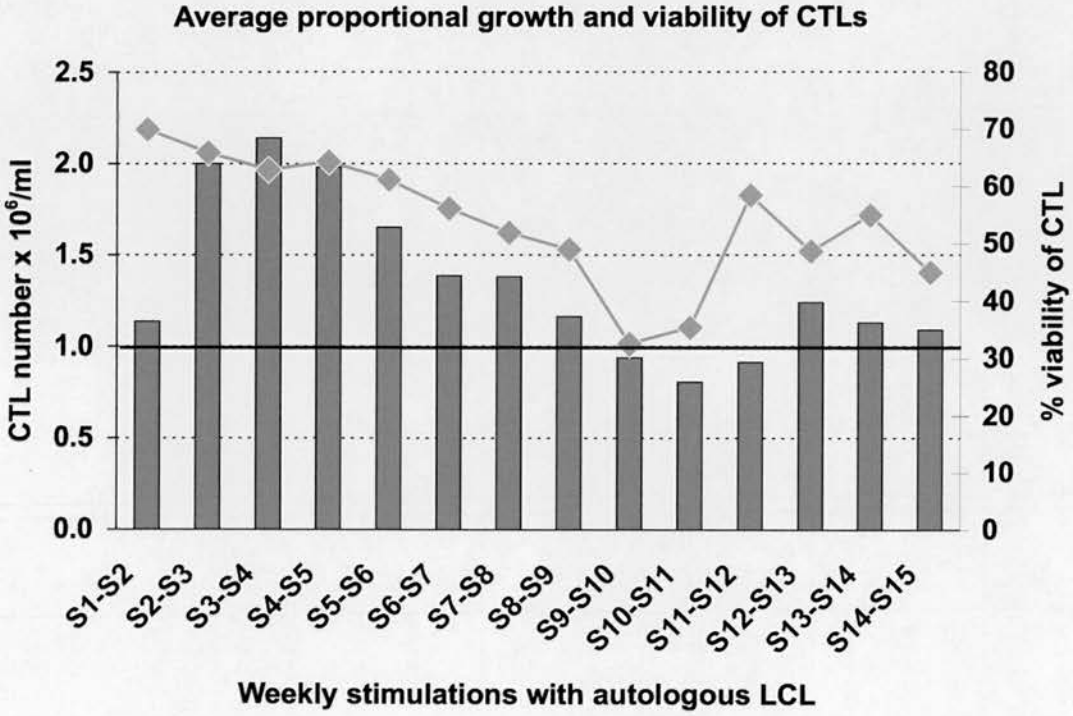


Figure 3.1: Average proportional growth (red bars) and viability (blue line) of CTLs at each weekly stimulation with autologous lymphoblastoid cells (LCLs). S: stimulation. See table 3.2 for data used to generate this figure.

The CTLs were grown in culture for up to 15 weeks, although some CTLs were not cultured for so long because cell numbers naturally decreased before that time and in other cases technical constraints halted the expansion of the cell lines. Figure 3.1 shows that there is a proportional increase in cell number from S1 to S4. From S4

onwards, there is a decrease in cell number week on week. This is paralleled by a fall in viability of the CTLs. From stimulations 10 to 15 there was break in the proportional growth and viability trends but fewer CTLs were growing over that period and therefore a low sample number may be responsible for this.

The data used to generate figure 3.1 is given in the table 3.2. The numbers in brackets in the percentage viability column are the numbers of CTLs for which these data were available. In the weekly stimulation column, "S" refers to stimulation.

Weekly stimulation	Average CTL number/ml of culture	% viability of CTL	Number of CTL lines growing
S1-S2	1.1×10^6 CTLs/ml	72% (11)	14
S2-S3	2.0×10^6 CTLs/ml	67% (13)	14
S3-S4	2.1×10^6 CTLs/ml	63% (13)	14
S4-S5	2.0×10^6 CTLs/ml	63% (13)	14
S5-S6	1.7×10^6 CTLs/ml	61% (13)	14
S6-S7	1.4×10^6 CTLs/ml	56% (12)	13
S7-S8	1.4×10^6 CTLs/ml	53% (9)	9
S8-S9	1.2×10^6 CTLs/ml	47% (4)	4
S9-S10	0.9×10^6 CTLs/ml	33% (2)	2
S10-S11	0.9×10^6 CTLs/ml	35% (3)	3
S11-S12	0.9×10^6 CTLs/ml	56% (2)	2
S12-S13	1.2×10^6 CTLs/ml	49% (3)	3
S13-S14	1.1×10^6 CTLs/ml	55% (1)	1
S14-S15	1.1×10^6 CTLs/ml	45% (1)	1

Table 3.2: Average CTL number/ml of culture, percentage of viable CTLs and the number of growing CTLs at each weekly stimulation. S: stimulation.

3.2.2 Basic phenotype of *ex vivo* generated CTLs

The basic phenotype of seven CTLs was investigated by FACS analysis. Based on the proportional growth trends of the CTLs as shown in figure 3.1, the analysis was divided into two phases – phase 1 from S1 to S4 where the proportional increase in CTL numbers continuously rose and peaked at S4 and phase 2 from S5 onwards where the proportional increase in CTL number fell. Basic phenotyping was based on CTL staining for CD4, CD8, CD19, CD45 and CD56 co-stained with CD3, as this is expressed only by T-cells. CD4 and CD8 staining was used to determine the phenotype of the T-cells; CD19 staining was used to detect B-cells and CD56 staining was used to detect NK cells. Staining with CD45 complemented staining with CD3 for the detection of T-cells, as all haematopoietic cells express CD45.

3.2.2.1 FACS analysis

FACS data were acquired for CTLs by gating on the lymphocytes based on their forward scatter and their side scatter. The forward scatter is a measure of the size of the cells and the side scatter is a measure of the granularity of the cells. Both the forward scatter and side scatter can be used to differentiate between live and dead cells, with dead cells having a lower forward scatter and higher side scatter than live cells. Based on this information, a gate was set around live lymphocytes (including T-cells) being acquired but also analysed, as shown in figures 3.2 a) and b). In figure 3.2 a) and b) every dot represents a cell, however in figure b) areas of high cell density can be identified (high cell density area appearing light red). The gate in figure 3.2 named R1 distinctly surrounds the live lymphocyte population (including T-cells) in figures 3.2 a) and b). The more diffuse and less dense population of cells, which is surrounded by blue lines, had a lower forward scatter and a higher side scatter indicating that these were dead cells. Figures 3.2 a) and b) represent the same CTL.

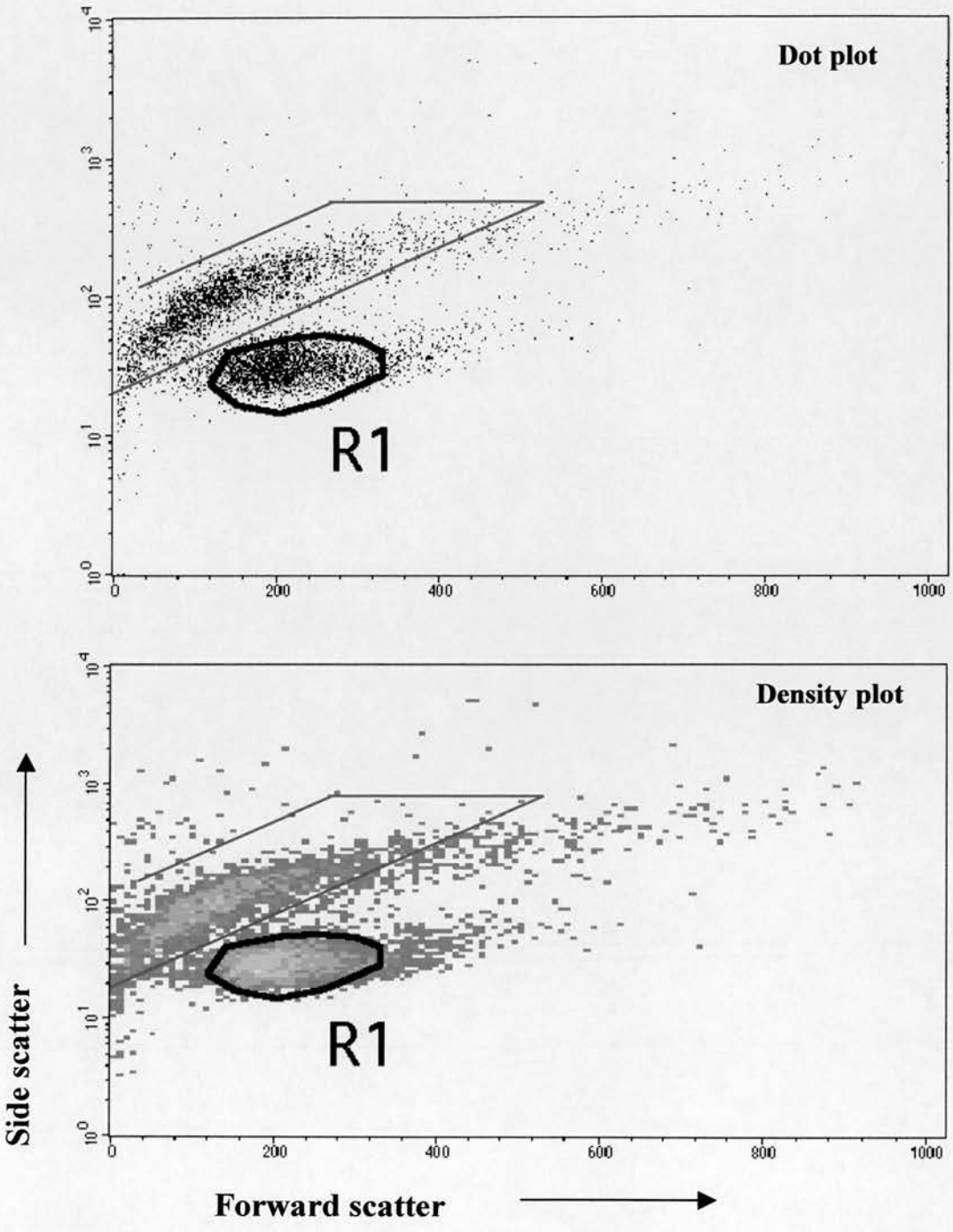


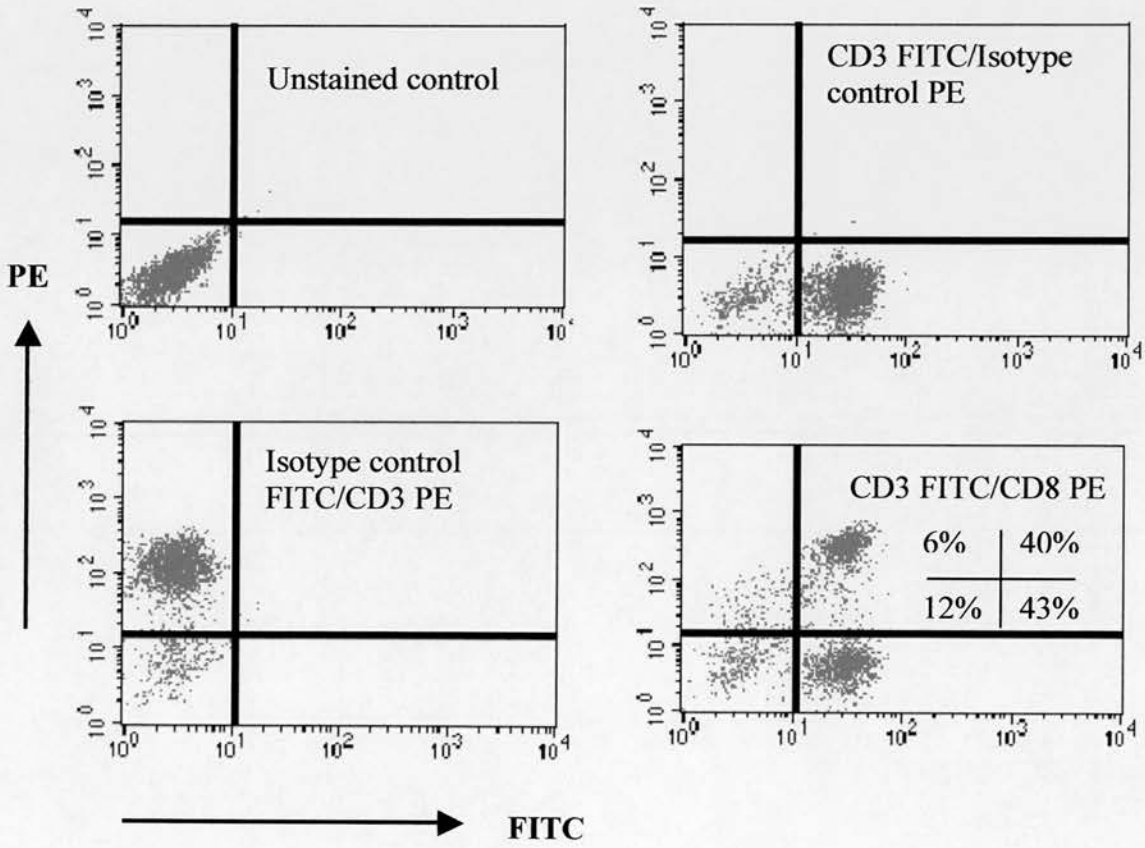
Figure 3.2: Identification of live lymphocytes within a CTL based on forward and side scatter in a) a dot plot and b) a density plot. Black gate R1– live lymphocytes. Blue gate – dead cells.

3.2.2.2 Basic phenotyping of *ex vivo* generated CTLs

Once this gate (shown as R1 in figure 3.2) was set for lymphocytes, the phenotype of these cells was determined by staining with fluorochrome-labelled antibodies specific for the extracellular markers discussed in section 3.1.2.

Figure 3.3 shows representative dot plots for CD3 and CD8 double staining obtained for one CTL in phases 1 and 2 of growth. The data shown in these dot plots as well other data collected for that CTL are given in table 3.3. Data for two other CTLs are also included in table 3.3.

a) CTL 14 at S1 (phase 1 of growth)



b) CTL 14 at stimulation 8 (phase 2 of growth)

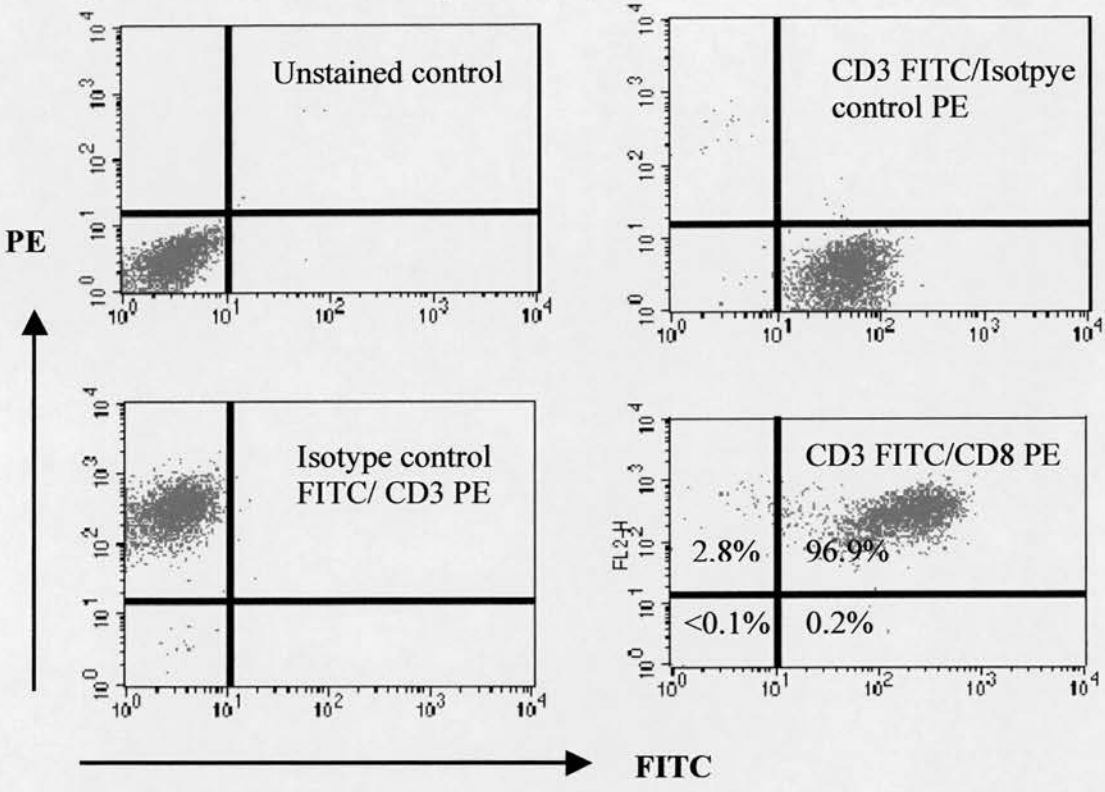


Figure 3.3: FACS analysis plots for CTL 14.

	Phase 1		Phase 2		
CTLs	**1**	**14**	**1**	**6**	**14**
CD4	15.6%	10%	2.7%	0.5%	5.8%
CD8	65.9%	43%	93.2%	97.8%	96.9%
CD19	0.2%	NA	0.3%	0.4%	0.1%
CD45	81.6%	NA	97%	98.5%	99.1%
CD56	1.4%	NA	1.9%	0.1%	1%

Table 3.3: Phenotype of CTLs 1, 6 (not phase 1) and 14. NA: not available.

The basic phenotype of four other CTLs, across phases 1 and 2 of growth, was determined and is shown in figures 3.4, 3.5, 3.6 and 3.7. Figures 3.4 to 3.7 show that the phenotype of the CTLs in phase 1 of growth persisted into phase 2. This was seen in all CTLs (whether the CTL became predominantly CD8+ (CTL 4 and CTL 3) or

whether it became predominantly CD4+ (CTL 13 and CTL 2)). These figures also show that in CTLs, which became predominantly CD4+ (CTLs 2 and 13) there was an initial decrease in the proportion of CD4+ T-cells followed by an increase at S3. This, though, was not seen in CTLs that became predominantly CD8+.

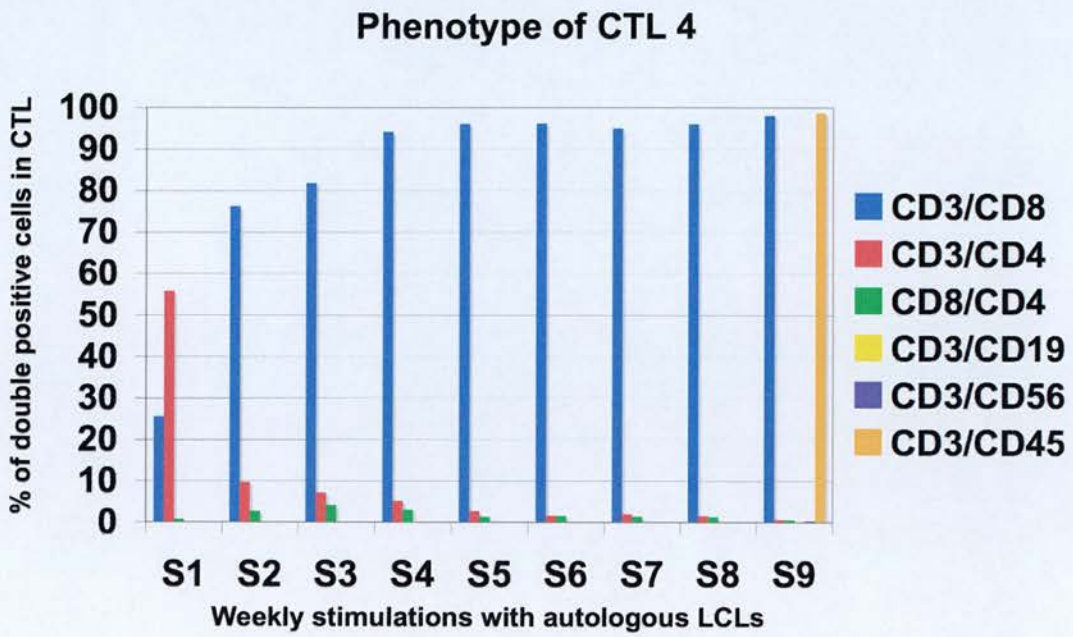


Figure 3.4: Phenotype of CTL 4.

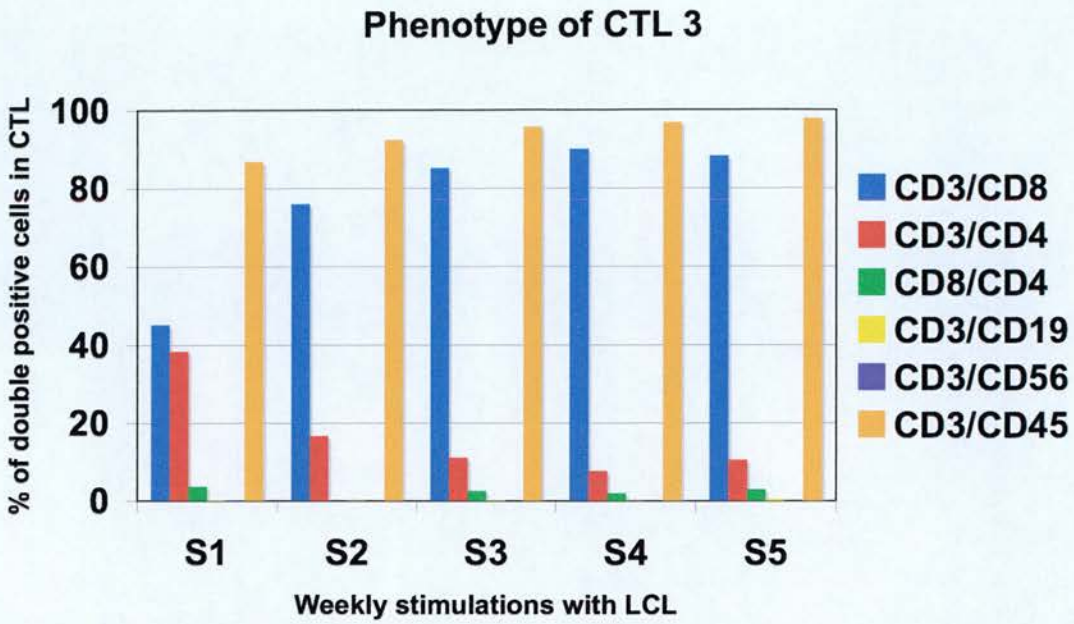


Figure 3.5: Phenotype of CTL 3.

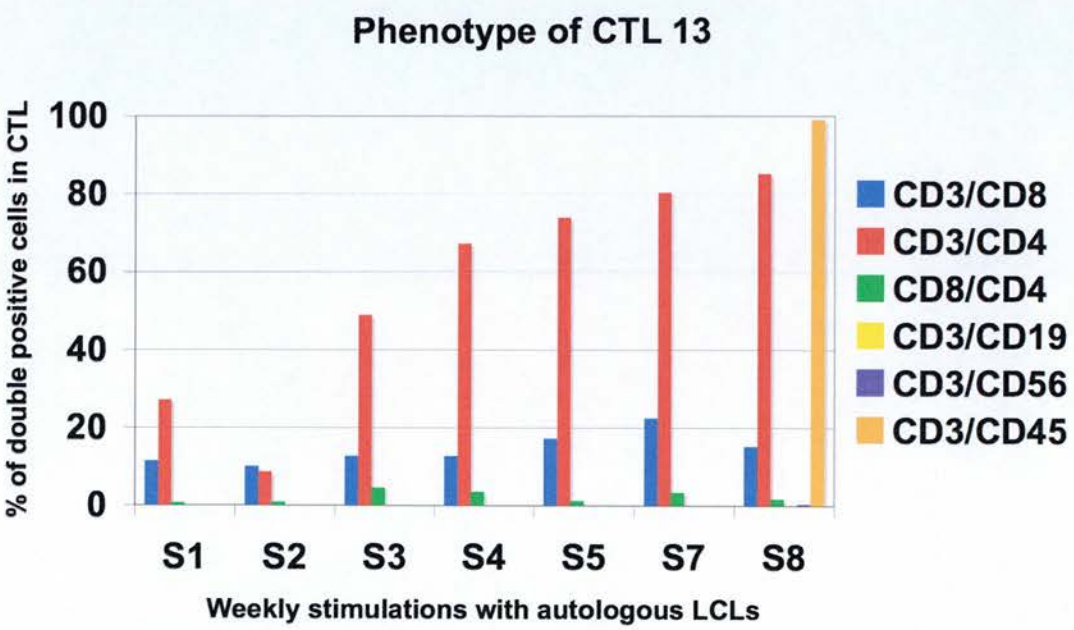


Figure 3.6 Phenotype of CTL 13.

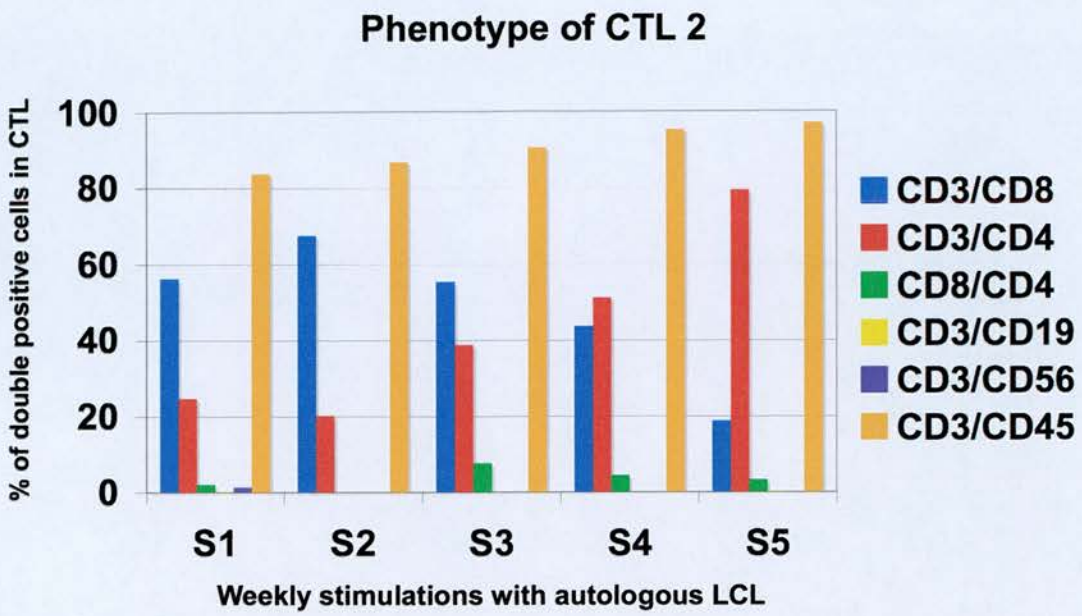


Figure 3.7: Phenotype of CTL 2.

In the CTLs which were phenotyped there was ≤2% CD56+ cells, <1% CD19+ B-cells and >97% of T-cells in phase 2 of growth. Where the CTL was predominantly CD4+ or CD8+ in phase 2, ≥79% of the CTLs expressed the dominant marker.

3.2.3 Extended phenotype of *ex vivo* generated CTLs

Having established the basic phenotype of the CTLs above, other CTLs were phenotyped more extensively by assessing the expression of activation and differentiation markers, such as CD27, CD28, CD45RA, CD45RO, CD62L and CD69. Phenotyping of CTLs was undertaken in growth phase 1 and phase 2. It was undertaken in phase 1 because phenotype determination, with regard to CD4 and CD8 expression, occurred during that phase. Therefore, the expression of the above-mentioned markers was investigated in conjunction with CD4 and CD8 expression. This was carried out to determine whether there was a pattern in phenotypic changes undergone by the CTLs that extended beyond CD4+ and CD8+ T-cell expression

and whether the same phenotypic changes occurred in CD4+ and CD8+ T-cells alike in the CTLs.

The categorisation of T-cells is a difficult issue with a wide variety of markers used to define particular cell subsets. CD45RO is a marker of antigen-experienced/mature cells. CD45RA is a marker of naïve T-cells, which is lost upon antigenic stimulation but can be re-expressed on late-differentiated cells. CD27 and CD28 are both T-cell co-stimulatory molecules, which are expressed on naïve T-cells. CD27 expression is upregulated upon antigenic stimulation of the TCR and it is subsequently downregulated (Hendriks *et al*, 2000). CD28 expression is downregulated upon TCR stimulation. Once CD27 or CD28 expression is lost neither marker can be re-expressed by either CD4+ T-cells or CD8+ T-cells, indicating that the cells differentiate into mature cells (Appay, 2004; Hendriks *et al*, 2000). Detection of these markers complements the detection of CD45RA to identify naïve T-cells in the CTLs. Additionally, the expression of CD62L, a lymphoid homing marker, was also investigated. It is expressed on both naïve CD4+ and CD8+ T-cells. Its expression is also downregulated upon antigenic stimulation. Finally, the expression of CD69, which is a marker of early T-cell activation, was also investigated.

3.2.3.1 Study of phenotype development of CTLs

The extended phenotype of two CTLs was studied in this part of the project. The basic phenotype of one CTL is shown in figure 3.8. This figure shows that by S4, in excess of 80% of the cells in the CTL expressed CD8 and fewer than 10% expressed CD4. It also shows that nearly 100% of the cells expressed CD3 after four weeks of culture.

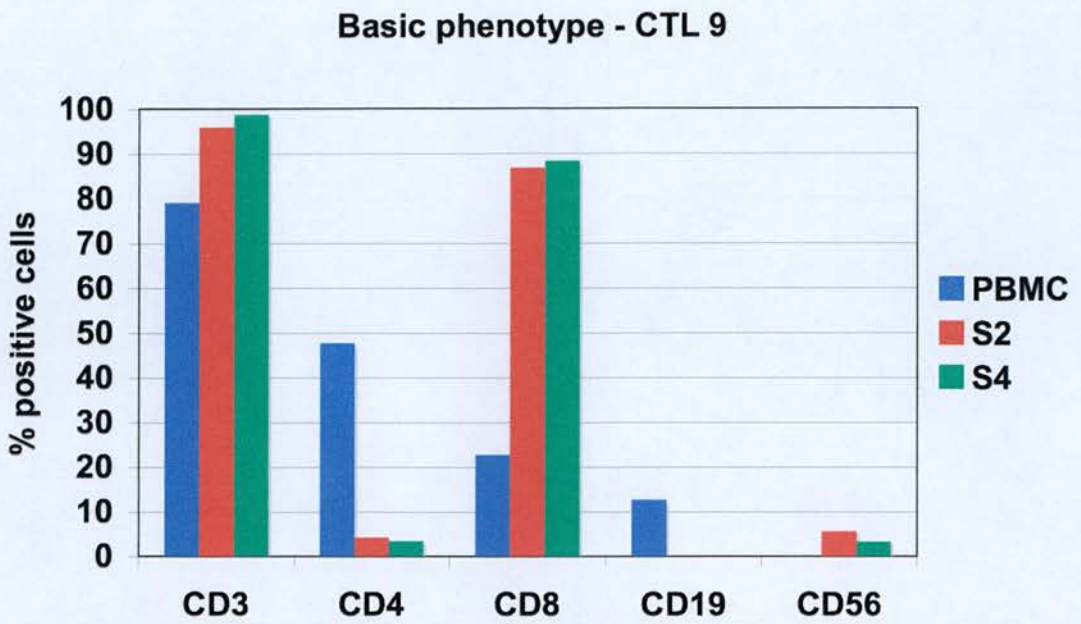


Figure 3.8: Basic phenotype of CTL 9.

The extended phenotype of the CTLs was established and is shown in figures 3.9 and 3.10. Figures 3.9 and 3.10 show that there was an overall decrease from stimulation 0 (PBMC) to S4 in the proportion cells co-expressing CD4 or CD8 and the naïve T-cell markers CD45RA, CD62L, CD27 and CD28. These figures also show that there was an overall increase in the proportion of cells co-expressing CD4 or CD8 and CD45RO (a marker of T-cell maturity) and CD69 (a marker of T-cell activation). This indicates that the CTL was, overall, progressing towards an antigen-experienced/mature phenotype.

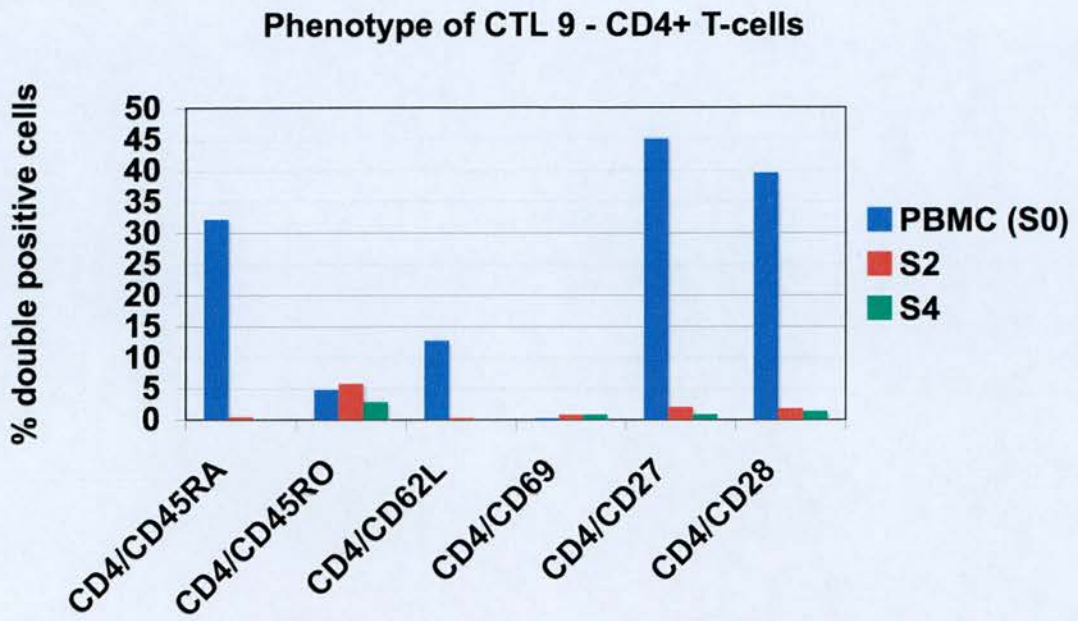


Figure 3.9: Phenotype of cells co-expressing CD4 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 9.

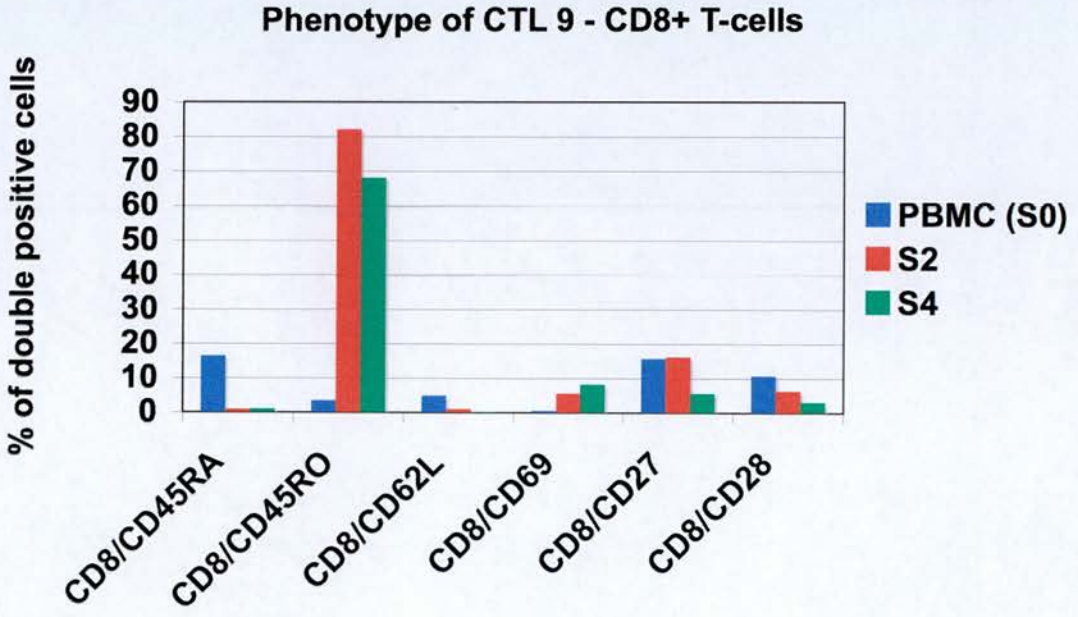


Figure 3.10: Phenotype of cells co-expressing CD8 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 9.

As the proportion of CD4+ T-cells and CD8+ T-cells in the CTL changed during the course of culture, the expression profile of CD4+ and CD8+ T-cells respectively was also investigated in order to determine whether similar phenotypic changes occurred in each cell type. The results of this are shown in figures 3.11 and 3.12. The proportion of CD4+ T-cells decreased and the proportion of CD8+ T-cells increased over time (figure 3.8). Figures 3.11 and 3.12 show that there was a decrease in the proportion of both CD4+ and CD8+ T-cells expressing CD45RA and CD62L, indicating that the cells were losing naïve marker expression. The expression of both CD27 and CD28 also decreased during the first four weeks of culture, with a greater decrease in expression in CD8+ T-cells. Conversely, the proportion of CD4+ T-cells and CD8+ T-cells expressing CD45RO increased dramatically, with over 90% of both cell types expressing this antigen-experienced/mature marker. These findings indicate that CD4+ T-cells were becoming antigen-experienced/mature cells even though their overall proportion in the CTL was decreasing (figure 3.8). The same applied to CD8+ T-cells, although their proportion increased over time in the CTL.

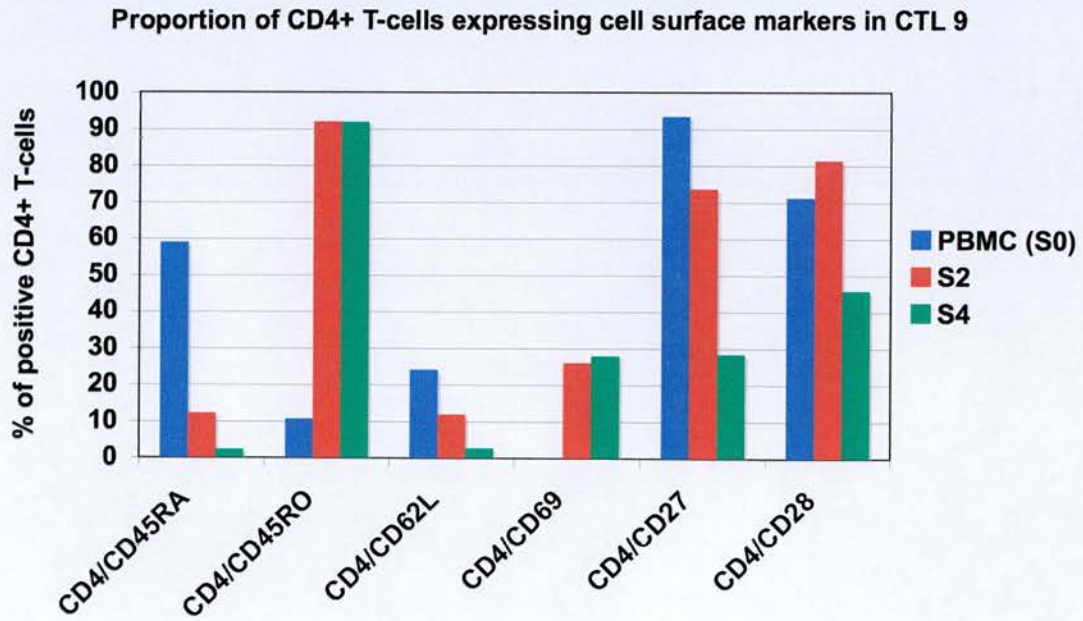


Figure 3.11: Proportion of CD4+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 9. This was determined by gating on CD4+ T-cells in the CTL.

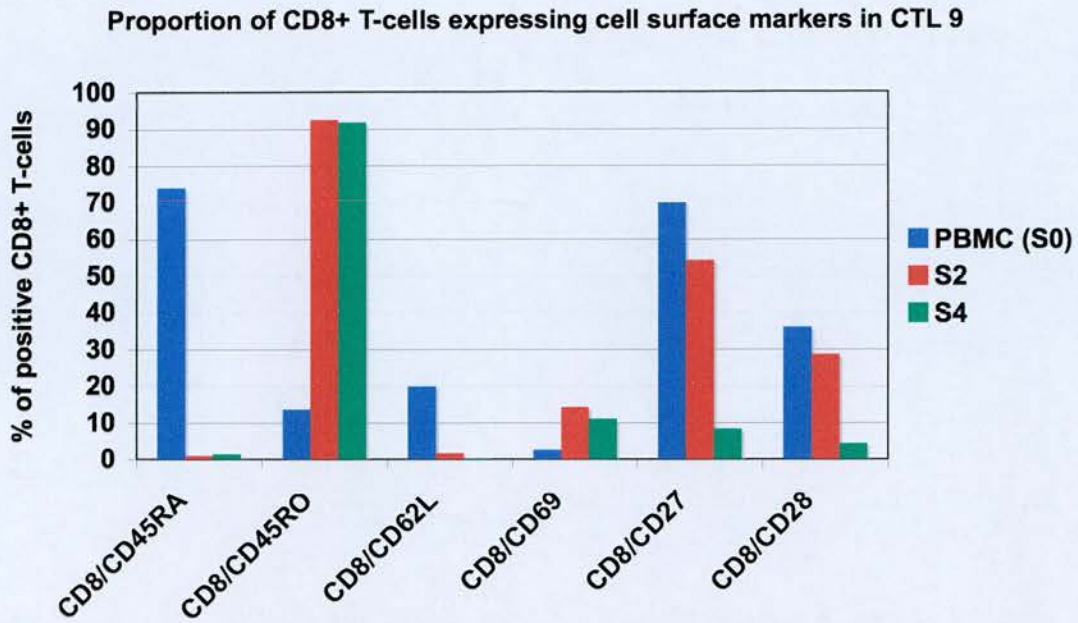


Figure 3.12: Proportion of CD8+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 9. This was determined by gating on CD8+ T-cells in the CTL.

The phenotypic pattern seen in figures 3.8 to 3.12 for CTL 9 was also seen in CTL 5, which was also immunophenotyped at stimulations 0, 2 and 4. Data for CTL 5 are shown in figures A3.1 to A3.5 in appendix 3.

The phenotype of two other CTLs (7 and 12) was established at stimulation 0 (PBMC) and in growth phase 2. Figures 3.13, 3.14 and 3.15 show the phenotype of CTL 12. These figures indicate that the phenotype observed in phase 1 of growth persisted into phase 2 of growth, with this cell line also displaying a mature phenotype. The data for CTL 7, which was phenotyped at stimulation 0 and at two stimulations in growth phase 2, are given in figures A3.6 to A3.10 in appendix A3. In this CTL the proportion of cells expressing both CD4 and CD8 increased from stimulation 0 (PBMC) to stimulations 5 and 6. Upregulation of CD4 on activated CD8+ T-cells has been documented (Sullivan *et al*, 2001).

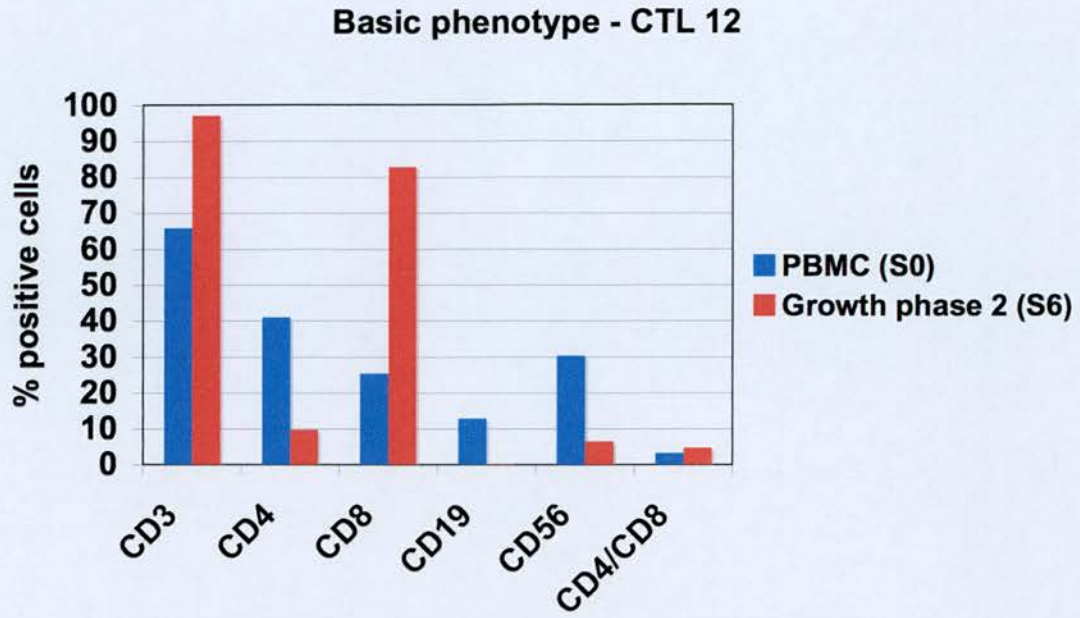


Figure 3.13: Basic phenotype of CTL 12.

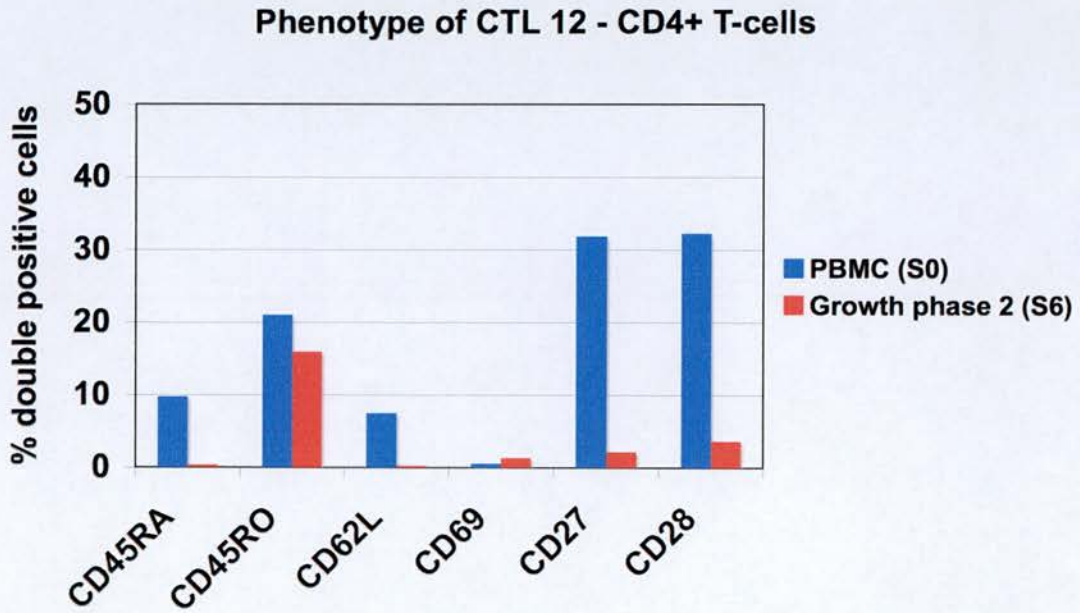


Figure 3.14: Phenotype of cells co-expressing CD4 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 12.

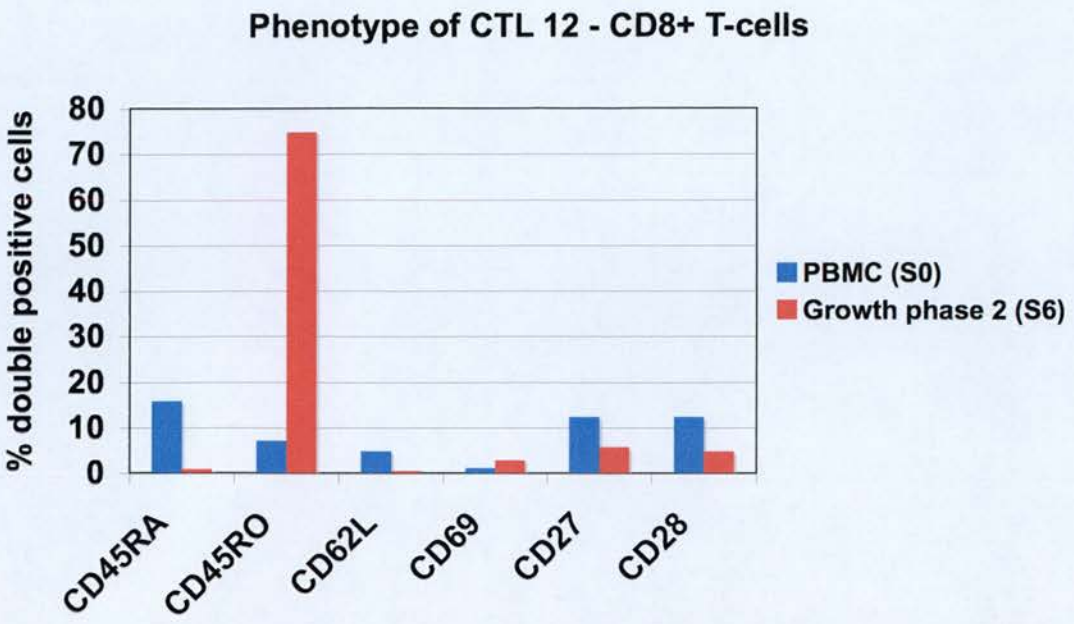


Figure 3.15: Phenotype of cells co-expressing CD8 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL12.

The phenotypic changes seen in the CD4+ and CD8+ T-cells of CTL 12 (in phase 2 of growth) are similar to those described for CTLs 5 and 9 (in phase 1 of growth). This is shown in figures 3.16 and 3.17. These findings indicate that CD4+ T-cells were becoming mature cells even though their overall proportion in the CTL was decreasing (figure 3.13). The same applied to CD8+ T-cells, although their proportion increased over time in the CTL.

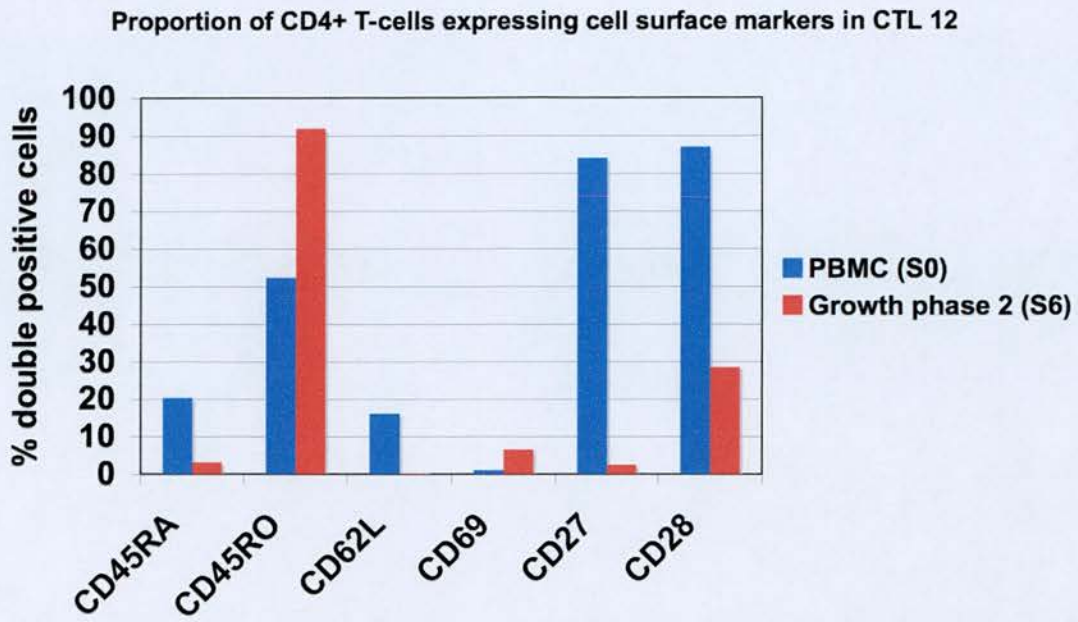


Figure 3.16: Proportion of CD4+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 12.

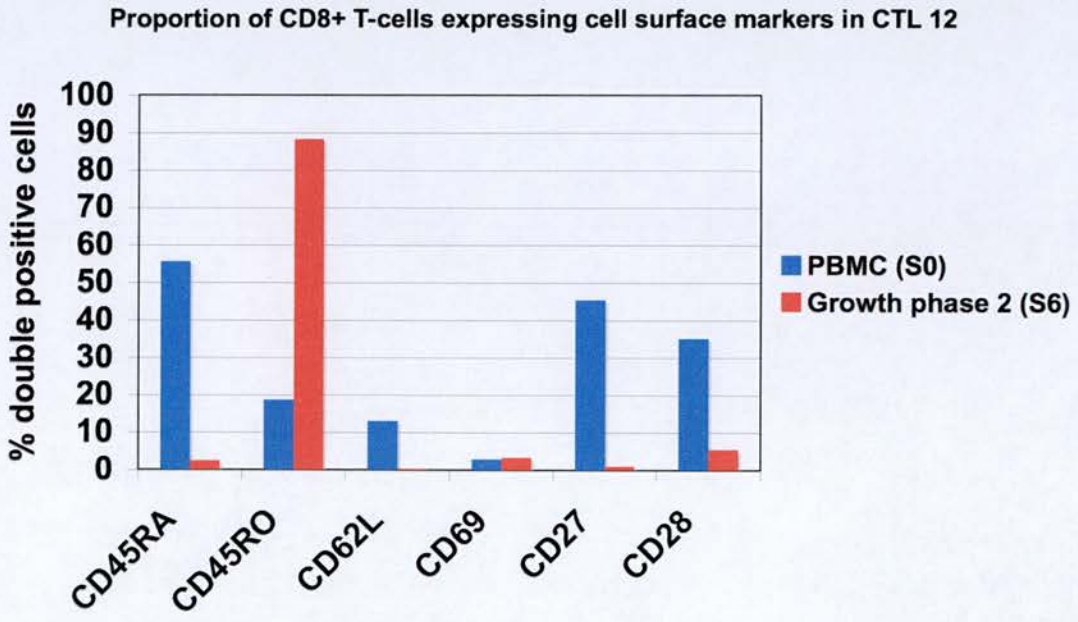


Figure 3.17: Proportion of CD8+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 12.

3.2.3.2 Summary of results on phenotypic development

These results indicate that beyond CD4 and CD8 phenotypic evolution described in section 3.2.2, there are extensive phenotypic changes occurring during the first 4 weeks of culture (growth phase 1), with these changes persisting in growth phase 2. The CTLs overall displayed a mature phenotype with decreased expression of CD45RA, CD62L, CD27 and CD28 and increased expression of CD45RO and CD69. The CD4+ and CD8+ T-cells within the CTLs also showed increased expression CD45RO and CD69 and a decrease in expression of CD45RA, CD62L, CD27 and CD28 indicating that both cell types were contributing to the overall mature phenotype of the CTLs.

3.3 Investigation into CD4+ T-cell and CD8+ T-cell interactions

It appears from the FACS analyses carried out on the CTLs that phase 1 is the key phase in terms of phenotypic development. In order to carry out basic investigations into potential polyclonal CD4+ T-cell and CD8+ T-cell interactions, three methods of positive cell selection were assessed.

3.3.1 EasySep, MACS and Dynal cell separation methods

Three different cell separation systems were trialled: EasySep, MACS and Dynal. All three of the methods were designed to positively select CD4+ T-cells from PBMCs. The detailed methods for each cell separation system are given in section 2.2. These three methods were first assessed using fresh PBMCs from three healthy laboratory volunteers. The method (EasySep) that yielded the largest fraction of enriched CD4+ T-cells was subsequently used on frozen PBMCs from three other donors. The purity of the positively selected enriched CD4+ T-cell fraction was assessed by FACS analysis. The percentage of total live cells recovered (from the CD4+ T-cell enriched fraction as well as CD4+ T-cell depleted supernatant) was determined by trypan blue exclusion cell counts.

3.3.1.1 Positive selection of CD4+ T-cells from fresh PBMCs

The purity of the positively selected CD4+ T-cell enriched fraction and the percentage of cells recovered post-selection for all three donors are summarised in figure 3.18. The purity of the positively selected CD4+ T-cell enriched fraction was comparable for all three methods: the EasySep method achieved on average 97% purity and the MACS and Dynal methods achieved on average 98% (98% MACS and 98.3% Dynal) purity based on the proportion of cells expressing CD3 and CD4. The percentage of cells recovered varied considerably with 66% of cells recovered

following EasySep separation, 52% following MACS separation and only 6% following Dynal separation.

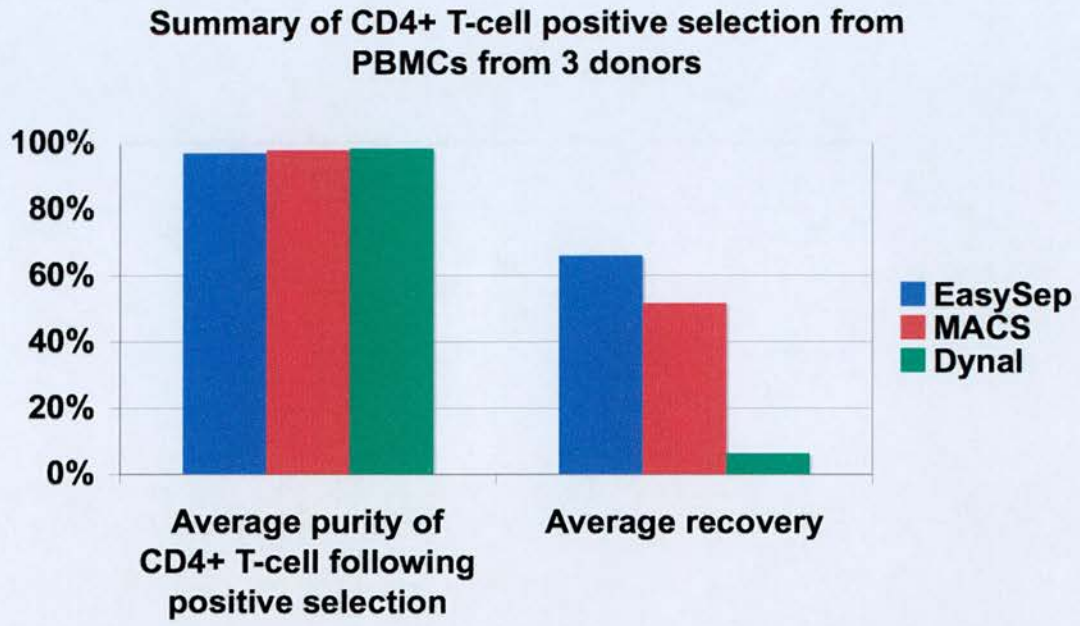


Figure 3.18: Summary of CD4+ T-cell positive selection from fresh PBMCs from three donors using EasySep, MACS and Dynal positive selection methods. (CD4+ T-cells were co-stained with CD3).

The presence of CD8+ T-cells within the positively selected CD4+ T-cell enriched fraction was also assessed by FACS analysis for all three donors and results are summarised in table 3.4.

% CD8+ T-cells	EasySep	MACS	Dynal
Donor A	2%	3%	1%
Donor B	2%	1%	1%
Donor C	10%	9%	5%
Mean % CD8+ T-cells	4.7%	4.3%	2.3%

Table 3.4: Percentage of CD8+ T-cells in the positively selected CD4+ T-cell enriched fraction.

The percentage of CD4+ T-cells remaining in the CD4+ T-cell depleted supernatant was also established and the results are given in table 3.5.

% CD4+ T-cells	EasySep	MACS	Dynal
Donor A	2%	2%	NA
Donor B	1%	3%	NA
Donor C	2%	3%	NA
Mean % CD4+ T-cells	1.6%	2.7%	NA

Table 3.5: Percentage of CD4+ T-cells remaining in the CD4+ T-cell depleted supernatant (NA: not available – not enough cells for analysis).

Additionally, the percentage of T-cells expressing both CD4 and CD8 was established as such cells may have been positively selected during each cell separation and may be responsible for part of the CD8+ T-cell contamination of the positively CD4+ T-cell enriched fraction seen in all three donors using all three cell separation methods (table 3.4). The percentages of T-cells positive for CD3+/CD4+, CD3+/CD8+ and CD4+/CD8+ in the PBMCs from each donor are given in table 3.6.

PBMC	CD3+/CD4+	CD3+/CD8+	CD4+/CD8+
Donor A	38%	33%	<1%
Donor B	30%	24%	1%
Donor C	28%	32%	1%

Table 3.6: Percentages of CD3+/CD4+, CD3+/CD8+ and CD4+/CD8+ T-cells in all three donors.

Based on the very low percentages of double positive CD4+/CD8+ T-cells in all three donors, these cells should not contribute to contamination of the positively selected CD4+ T-cell enriched fraction with CD3+/CD8+ T-cells. In order to confirm this the percentages of double positive CD4+/CD8+ T-cells in the CD4+ T-cell enriched fraction following CD4+ T-cell positive selection were established and are given in table 3.7.

CD4+/CD8+ T-cells	EasySep	MACS	Dynal
Donor A	1%	1%	NA
Donor B	1%	1%	<1%
Donor C	6%	3%	5%

Table 3.7: Percentage of double positive CD4+/CD8+ T-cells in the CD4+ T-cell enriched fraction. NA: not available - not enough cells.

The percentage of double positive CD4+/CD8+ T-cells in the CD4+ T-cell enriched fraction following all three separation methods were similar to those seen in PBMCs (table 3.6) for donors A and B. This suggests that double positive CD4+/CD8+ T-cells did not form part of the CD3+/CD8+ T-cell contaminating fraction (table 3.7) in these donors. However, following CD4+ T-cell positive selection this percentage was elevated in donor C compared with the percentage seen in PBMCs. The percentage of double positive CD4+/CD8+ T-cells in donor C though remained lower than the percentage of double positive CD3+/CD8+ T-cells following all three cell separation methods as shown in table 3.7. It may therefore be posited that double positive CD4+/CD8+ T-cells are part of the overall contaminating CD3+/CD8+ T-cell fraction and may also be expressing CD3.

3.3.1.2 Summary of CD4+ T-cell positive selection methods on fresh PBMCs

Based on the results obtained above, the Dynal separation method was not considered for further experiments due to the poor recovery of cells post-separation, even though the CD4+ T-cell enriched fraction in all three donors was on average 98% pure. The MACS separation method also yielded on average a 98% pure CD4+ T-cell enriched fraction as well as a far greater total cell recovery than the Dynal separation method (MACS 52% vs Dynal 6%). The EasySep separation method yielded on average a 97% pure CD4+ T-cell enriched fraction but a total cell recovery of 66%, which was greater than with the MACS and Dynal separation methods. Additionally the EasySep separation method was technically more

straightforward than the two other methods. The EasySep separation method was therefore tested further on frozen PBMCs.

3.3.2 Positive selection of CD4+ T-cells from frozen PBMCs using EasySep

The EasySep method was used to positively select CD4+ T-cells from frozen PBMCs from two donors. The results of these positive selections are given in table 3.8.

	% CD4+ T-cells	% CD8+ T-cells	% cells recovered
Donor 1	72%	27%	26%
Donor 2	96%	<1%	38%

Table 3.8: Summary of purity of the CD4+ T-cell enriched fraction and the percentage of cells recovered post-separation.

The purity of the CD4+ T-cell enriched fraction for donor 2 was in line with the results obtained using the fresh PBMCs. However, the purity for donor 1 was lower with only 72% of CD4+ T-cells and 27% of contaminating CD8+ T-cells. Furthermore, the percentage of cells recovered from both donors was poor. This was due to the cell clumping during the separation procedure.

3.3.3 Summary of cell separation trial

The enrichment of CD8+ T-cells from PBMCs (fresh or frozen) was not investigated, as this uses the same method as CD4+ T-cell enrichment. The results of the CD4+ T-cell enrichment trials were not adequate for the downstream experiments planned. Another method would have to be developed in order to investigate CD4+ T-cell and CD8+ T-cell interactions in CTL development, but this was not carried out as part of this project.

3.4 Discussion

3.4.1 Growth of CTLs

Cytotoxic T-cell lines have been generated and used in the treatment of PTLD. Such CTLs were shown to expand up to $2x10^9$ cells over the course of 10 weeks of culture (Wilkie *et al*, 2004). The pattern of proportional growth of the CTLs generated in this project was the same as previously reported (Wilkie *et al*, 2004). The CTLs proportionally increased between S2 and S4, after which numbers decreased. The viability of the CTLs decreased from S2 onwards and decreased more rapidly after S4. Proportional growth is likely to occur as activation-induced cell death is overcome by the addition of IL-2, which maintains T-cell proliferation (Seder and Ahmed, 2003). The growth trend of the CTLs was established as this may provide an indication for the mechanism that limits CTL proliferation and therefore suggests ways by which this may be manipulated.

There would appear to be a limit to the potential growth that the CTLs could achieve. *In vitro* mitogen-stimulated CD8+ T-cells express a protein, $p16^{INK4a}$, essential in cell proliferation control (Migliaccio *et al*, 2005). Expression of this protein is associated with cessation of proliferation of a large proportion of *in vitro* mitogen-stimulated CD8+ T-cells. The CD8+ T-cells were stimulated every 10 to 15 days and upon the fifth round of stimulation the greatest proportion of cells expressing $p16^{INK4a}$ was observed indicating that the greatest proportion of cells exiting the cell proliferation cycle occurred at that time. A parallel may potentially be drawn between this finding and the fact that the CTLs grown in this project showed a decrease in proportional growth following five weeks of culture, equivalent to five rounds of stimulation. However, the CTLs generated here, as well as those characterised by Wilkie *et al* (2004) were not purified CD8+ T-cell cultures, although the majority of these CTLs comprised >80% of CD8+ T-cells.

Another potential factor limiting the period of *in vitro* CTL growth may be related replicative senescence. Replicative senescence occurs when telomere shortening becomes critical and it is no longer possible for the cell to divide appropriately, at which point cell cycle arrest occurs. Transduction of human telomerase reverse transcriptase, which can reverse the effects of replicative senescence, into CD8+ T-cells specific for melanoma antigen recognised by T-cell 1 (MART-1) resulted in the ability to culture these CD8+ T-cells for at least 10 months (Schreurs *et al*, 2005). However, following 12 months of culture these cells progressed into immunosenescence where functional activity was lost. This was in stark contrast to untransduced CD8+ T-cells whose lifespan was limited to three to four months, which is in line with the lifespan of the CTLs generated here.

3.4.2 Basic phenotype of the CTLs

There was a contrast between the two CD4+ predominant CTLs (2 and 13, in which there was an initial decrease in CD4+ T-cells followed by an increase) and CD8+ predominant CTLs (which showed a steady increase in proportion of CD8+ T-cells). This may due to the period of antigen exposure required to initiate the proliferation of naïve CD4+ T-cells, which appears to be longer than for CD8+ T-cells. Additionally, it may be due CD8+ T-cells dividing earlier and at a faster rate than CD4+ T-cells following exposure to antigen. This may be linked to fundamental differences in the differentiation and function undertaken by both cell types (Seder and Ahmed, 2003). These differences may also underlie the fact that the majority (82% - 9 out of 11 CTLs) of CTLs generated in this project were predominantly CD8+. Where the CTL was predominantly CD4+ or CD8+ in phase 2 of growth ≥79% of the CTLs expressed the dominant marker.

3.4.3 Phenotype of the CTLs

The results of extended phenotyping show that during the first four weeks of culture the phenotype of the CTLs progressed from a naïve phenotype seen in PBMC

(stimulation 0) to an antigen-experienced/mature phenotype. Additionally, the phenotype observed in phase 1 persisted in phase 2. This indicates that there is a pattern in the phenotypic development of the CTLs in response to antigen stimulation.

The increase in CD4+ T-cells and CD8+ T-cells co-expressing CD45RO in the CTLs and the concurrent decrease in CD4+ T-cells and CD8+ T-cells co-expressing CD45RA observed at S2 indicates that both cell types are responding to antigenic stimulation of the TCR and that both cell types are progressing towards an antigen-experienced/mature phenotype (Figures 3.9, 3.10 and A3.2 and A3.3). The increase in the proportion of cells expressing CD45RO was comparable in the two cell types, as was the decrease in the proportion of cells expressing CD45RA indicating that the cells had responded to antigen exposure. Progression towards an antigen-experienced/mature phenotype was also supported by a gradual decrease in CD4+ and CD8+ T-cells co-expressing CD27 or CD28 in the CTLs. However, the decrease in expression of both CD27 and CD28 was slower in CD4+ T-cells than in CD8+ T-cells. The proportions of CD4+ T-cells expressing either markers at stimulations 2 and 4 were higher than those observed for CD8+ T-cells. This may indicate that CD4+ T-cells in the CTLs differentiate more slowly in response to antigenic TCR stimulation. The progressive loss of these markers in CTLs *in vivo* is paralleled with the acquisition of cytolytic proteins, such as perforin, in both cell types, which would suggest that CD4+ T-cells acquire cytolytic potential later on in the culture period (Appay, 2004; Appay *et al*, 2002a). In conjunction with the gradual loss of CD27 and CD28 in the CTLs there was also a decrease in CD4+ T-cells and CD8+ T-cells co-expressing CD62L in the CTLs. This drop occurred during the first four weeks of culture, which indicates that both cell types were progressing towards an antigen-experienced/mature phenotype. A decrease in CD62L, on both CD4+ and CD8+ T-cells, would be critical in the context of PTLD immunotherapy as it is essential for the CTLs to home to the tumour and not to the lymph nodes. There was an increase in the proportion of CD4+ T-cells and CD8+ T-cells co-expressing CD69 in the CTLs indicating that the T-cells were activated following antigen exposure.

Based on these findings it would appear that weekly stimulations with autologous LCLs generate CTLs which display all the extracellular hallmarks of antigen-experienced/mature cells and that there is a pattern in CTL development beyond CD4+ and CD8+ expression. There were, however, differences between CD4+ T-cell and CD8+ T-cell differentiation, with CD8+ T-cells not only responding sooner to antigenic stimulation of the TCR, but also differentiating faster than CD4+ T-cells. As TCR stimulation is the key to the development of the CTLs in terms of growth and phenotypic evolution, investigation in the development of the TCR was carried out next, with a view to determine whether changes in TCR development also occurred. Additionally, the presence of cytolytic molecules such as perforin was investigated (to establish whether the CTLs were acquiring cytolytic molecules) and the results are presented in chapter 5.

Chapter 4

T-cell receptor spectratyping of CTLs

4.1 Introduction

4.2 T-cell receptor Vβ families in the CTLs

4.3 Evolution and clonality of Vβ families in CTLs

4.4 Development of T-cell clones within Vβ families

4.5 Discussion

4.1 Introduction

The T-cell receptor (TCR) is an immunoglobulin-like heterdomeric protein. It can be composed of membrane-bound α and β or γ and δ glycopeptide chains. Each chain is encoded by a series of variable (V), diversity (D), joining (J) and constant (C) gene segments. Recombination of these segments (V-J in the α chain and V-D-J in the β chain), as well as imprecise joining, random mutations and nucleotide insertion result in a wide variety of TCRs being generated. Variability in the TCR is concentrated around three complementarity determining-regions (CDRs). Complementarity determining-regions 1 and 2 are encoded by germline DNA in the V gene segments whereas CDR3 is formed by V-D-J joining and is more variable. The CDR3 of the TCR β chain is the most diverse (Sourdive *et al*, 1998). It is encoded in part by the variable β chain segments of DNA, and in part arises from imprecise joining, random mutations and nucleotide insertions. The CDRs are essential in binding the antigenic peptide/major histocompatibility complex (MHC), with CDR1 and CDR2 primarily binding the MHC and CDR3 binding antigenic peptide. CDR3 length is therefore used to assess diversity of the TCR.

The diversity of the TCR repertoire in CTLs stimulated weekly with autologous LCL was investigated by TCR spectratyping, an RT-PCR-based technique that measures the length of the CDR3. This was done by using 23 pairs of primers, specific for known sequences of variable β (Vβ) chain segments, the products of which are referred to as Vβ families. Products within these families vary in length within a given size range and identify different CDR3s. The TCR repertoire can become skewed following antigen exposure, with the preferential use of particular Vβ families (Kolowos *et al*, 1999).

CTLs are already in clinical use for PTLD treatment (Haque *et al*, 2001; Haque *et al*, 2002; Wilkie *et al*, 2004), however the diversity of their TCR repertoire has not been established. TCR spectratyping was therefore carried out on CTLs. Using TCR sepctratyping the number and the frequency of Vβ families in use in the CTLs during

the period of culture were established. Further investigations were carried out to determine the clonality of the Vβ families and how this evolved during culture time. Finally, the persistence of specific clones within polyclonal and clonal/biclonal families over time was studied.

4.2 T-cell receptor Vβ families in the CTLs

4.2.1 Cytotoxic T-cell lines

T-cell receptor sprectratyping was carried on CTLs across phases 1 and 2 of growth. Details of the CTLs analysed are given in table 4.1 where "S" refers to the weekly stimulation with autologous LCLs. Additionally, TCR spectratyping was carried out on the PBMCs used to generate CTLs 3, 4, 5 and 7.

	Stimulations at which TCR spectratyping was carried out	
CTLs	**Phase 1 of growth (S1 to S4)**	**Phase 2 of growth (S5 – onwards)**
2	S2, S3, S4	S5
3	S3	S5
4	S4	S5, S6, S7, S8, S9
5	S1, S2, S3, S4	S5, S6
7	S1, S2, S3, S4	S5, S6, S7, S8, S15
9	S1, S2, S3, S4	S5, S6
12	S2, S4	S5, S6, S7
13	S4	S5, S7, S8

Table 4.1: CTLs whose TCR was spectratyped. S: weekly stimulation with autologous LCL.

4.2.2 T-cell receptor spectratyping

T-cell receptor reverse-transcriptase polymerase chain reaction (TCR RT-PCR) and sequencing were carried out as described in section 2.3.4 of chapter 2. Figure 4.1 represents a typical agarose gel of TCR RT-PCR on one CTL obtained before sequencing. The TCR RT-PCR for CTLs 5 (S1 to S6), 7 (S1 to S8) and 9 (S1 to S6) were carried out by Karen McAulay.

1. 100bp ladder	5. Vβ-4 334-346 bp	9. Vβ-8 355-373 bp	13. Vβ-13 408-425 bp	17. Vβ-17 226-241 bp	21. Vβ-22 234-252 bp
2. Vβ-1 195-207 bp	6. Vβ-5 354-375 bp	10. Vβ-9 194-212 bp	14. Vβ-14 328-383 bp	18. Vβ-18 325-337 bp	22. Vβ-23 358-370 bp
3. Vβ-2 195-207 bp	7. Vβ-6 329-347 bp	11. Vβ-11 321-333 bp	15. Vβ-15 193-208 bp	19. Vβ-20 218-227 bp	23. Vβ-24 353-368 bp
4. Vβ-3 190-208 bp	8. Vβ-7 190-214 bp	12. Vβ-12 267-290 bp	16. Vβ-16 241-256 bp	20. Vβ-21 185-200 bp	24. Vβ-25 226-241 bp

Figure 4.1: Agarose gel of TCR RT-PCR on one CTL at one stimulation and table of expected product sizes. bp: base pair.

The data generated following sequencing of the RT-PCR products visualised on agarose gels, as seen in figure 4.1 are presented in graph form. One graph was generated for each Vβ family. A typical graph of one Vβ family is shown in figure 4.2. This is family Vβ-5 and the size of the RT-PCR products can range from 354bp to 375bp.

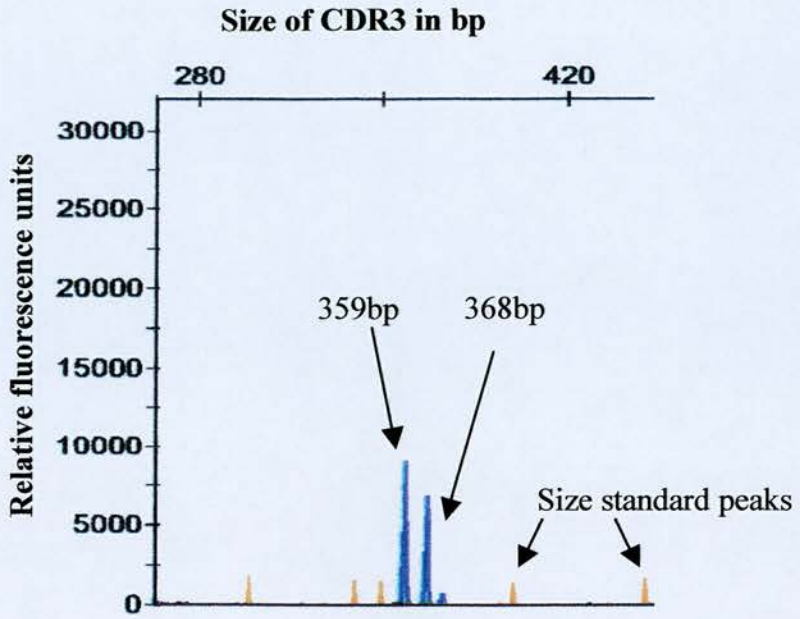


Figure 4.2: Graphical representation of one Vβ family. Blue peaks: CDR3 length and orange peaks: size standard.

In figure 4.2, there are two peaks, each corresponding to a T-cell clone with a particular CDR3 length for that Vβ family (359bp and 368bp). The difference between each peak is approximately a multiple of 3bp, which corresponds to one amino acid change in the CDR3. The relative fluorescence units (RFU) correspond to the relative expression of the T-cell clone in question within the CTL. Peaks with a RFU <1000 were considered as background.

4.2.3 Vβ family usage by the CTLs

The usage of Vβ families by the CTLs in the different phases of growth (including PBMCs) was investigated, as CTL usage of Vβ families had not been previously established. The usage of Vβ families by one CTL is summarised in table 4.2. Table 4.2 shows that the number of families used by the CTL varied from week to week but over the six-week culture period it shows that all Vβ families had been used with the exception of Vβ-25. The same was seen in the CTLs for which data are given in

appendix A4. Such tables were drawn up for all CTLs and are included in appendix A4.

CTL 9	Nb of families used	Families used
PBMC	14	2,3,4,5,6,9,11,12,15,16,17,21,22,23,24
S1	9	2,3,5,7,8,9,12,15,17,20,21
S2	19	1,2,3,4,5,6,7,8,9,11,12,13,14,15,17,18,20,21,22
S3	20	1,2,3,4,5,6,7,8,9,11,12,13,15,16,17,18,21,22,23,24
S4	18	1,3,4,5,6,7,8,9,12,13,14,15,16,17,18,21,22,23
S5	11	1,3,4,5,6,8,9,12,14,21,22
S6	16	1,2,3,4,5,6,7,8,11,12,14,15,16,17,21,22
Average nb of families used	15.3	All families were used over the course of six stimulations with LCLs, except Vβ-25

Table 4.2: Vβ families used by one CTL during six weeks of culture with weekly stimulations with LCLs. Nb: number.

The percentage of Vβ family usage by all CTLs at each stimulation analysed (given in table 4.1) is summarised in table 4.3 (i.e.: 100% means that a Vβ family was used by a CTL at each stimulation analysed and 50% means that a Vβ family was used in half of the stimulations analysed). This table shows that three of the CTLs used all of the Vβ families during the course of culture and five did not. Family Vβ-18 was not used by CTL 3, family Vβ-20 was not used by CTLs 3, 4 and 5 and family Vβ-25 was not used by CTLs 3, 4, 9 and 13. It also shows that the most frequently used Vβ family is Vβ-3, which was used by all CTLs at all weekly stimulations. Vβ families 2, 4, 5, 6, 9, 12, 21 and 22 were also frequently used, with an average of more than 80% of the CTLs using these families during culture. Other families were, on average, not as frequently used but this varied from one CTL to another, as seen with family Vβ-20 that was used by two CTLs at all stimulations analysed but was not used at all by three other CTLs. This indicated that Vβ family usage varies between CTLs but also within CTLs over time (table 4.2 and tables A4.1 to A4.7).

Vβ families	CTLs								Avg %
	2	3	4	5	7	8	12	13	
Vβ-1	75%	33%	57%	57%	80%	71%	60%	25%	**57%**
Vβ-2	100%	100%	100%	71%	78%	71%	100%	75%	**87%**
Vβ-3	100%	100%	100%	100%	100%	100%	100%	100%	**100%**
Vβ-4	75%	66%	100%	83%	100%	86%	100%	75%	**86%**
Vβ-5	50%	100%	100%	71%	80%	100%	100%	75%	**85%**
Vβ-6	75%	66%	100%	57%	100%	86%	80%	100%	**83%**
Vβ-7	75%	33%	50%	100%	67%	71%	80%	100%	**72%**
Vβ-8	75%	33%	100%	86%	90%	71%	80%	75%	**76%**
Vβ-9	100%	100%	86%	86%	100%	86%	80%	100%	**92%**
Vβ-11	100%	66%	29%	43%	60%	57%	40%	50%	**48%**
Vβ-12	100%	100%	100%	29%	70%	100%	100%	100%	**87%**
Vβ-13	75%	66%	100%	57%	70%	43%	60%	100%	**71%**
Vβ-14	75%	33%	100%	57%	80%	57%	100%	33%	**67%**
Vβ-15	75%	100%	71%	57%	90%	86%	80%	33%	**74%**
Vβ-16	100%	33%	57%	86%	90%	57%	100%	50%	**60%**
Vβ-17	75%	66%	57%	100%	70%	86%	60%	75%	**61%**
Vβ-18	25%	0%	14%	17%	60%	43%	20%	50%	**29%**
Vβ-20	100%	0%	0%	0%	70%	29%	60%	100%	**45%**
Vβ-21	100%	66%	100%	40%	80%	100%	100%	100%	**86%**
Vβ-22	100%	66%	83%	83%	89%	86%	100%	100%	**88%**
Vβ-23	50%	66%	86%	86%	78%	43%	80%	50%	**67%**
Vβ-24	100%	33%	43%	66%	78%	29%	40%	50%	**55%**
Vβ-25	25%	0%	0%	66%	20%	0%	20%	0%	**16%**

Table 4.3: Frequency of Vβ family use by all the CTLs over the course of their culture period.

Having found that the TCR repertoire of the CTLs was broad and hence diverse in terms of Vβ family usage during the course of culture families, the clonality of the families was investigated next.

4.3 Evolution and clonality of Vβ families in CTLs

4.3.1 Definition of clonal, biclonal and polyclonal Vβ families

The clonality of all the Vβ families used by the CTLs was investigated next, in order to determine whether the Vβ families evolved during the course of culture. Families were described either as polyclonal, biclonal or clonal depending on the number of peaks shown on the analysis graphs. The number of peaks corresponds to the number of T-cell clones expressing CDR3s from a family. Figure 4.3 shows a) a clonal family, where there is only one peak (one T-cell clone), b) a biclonal family, where there are two peaks (two T-cell clones with CDR3s of different lengths derived from the same Vβ family) and c) a polyclonal family, where there are in excess of two peaks (more than two T-cell clones with CDR3s of different lengths derived from the same Vβ family). Clonal and biclonal families were grouped for the purpose of analysis, as these are quite distinct from polyclonal families.

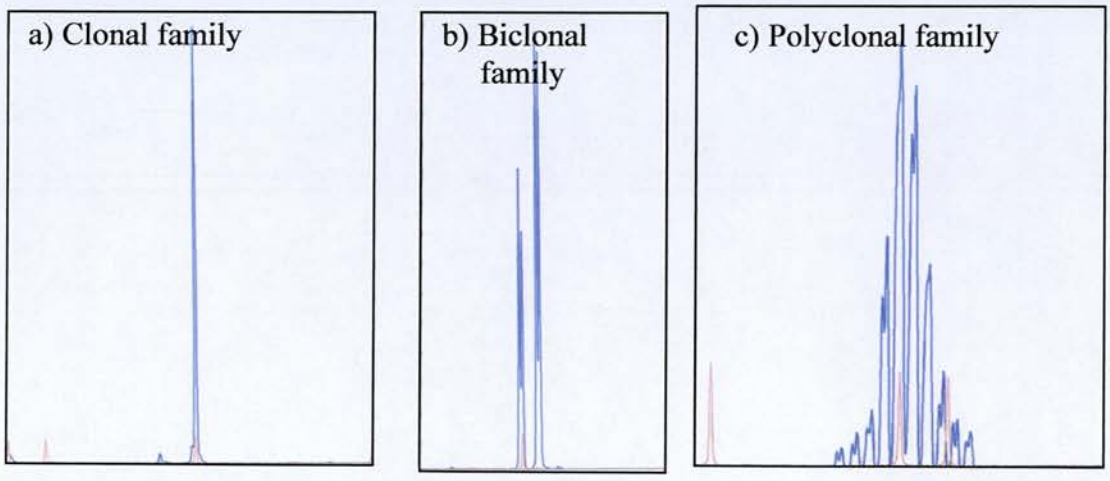


Figure 4.3: Definition of clonal, biclonal and polyclonal families.

4.3.2 Evolution and clonality of Vβ families in CTLs

The evolution of the Vβ families in the CTLs was established, with regard to the number of clonal/biclonal and polyclonal families being used by the CTLs at each weekly stimulation. Figure 4.4 shows the evolution of one CTL.

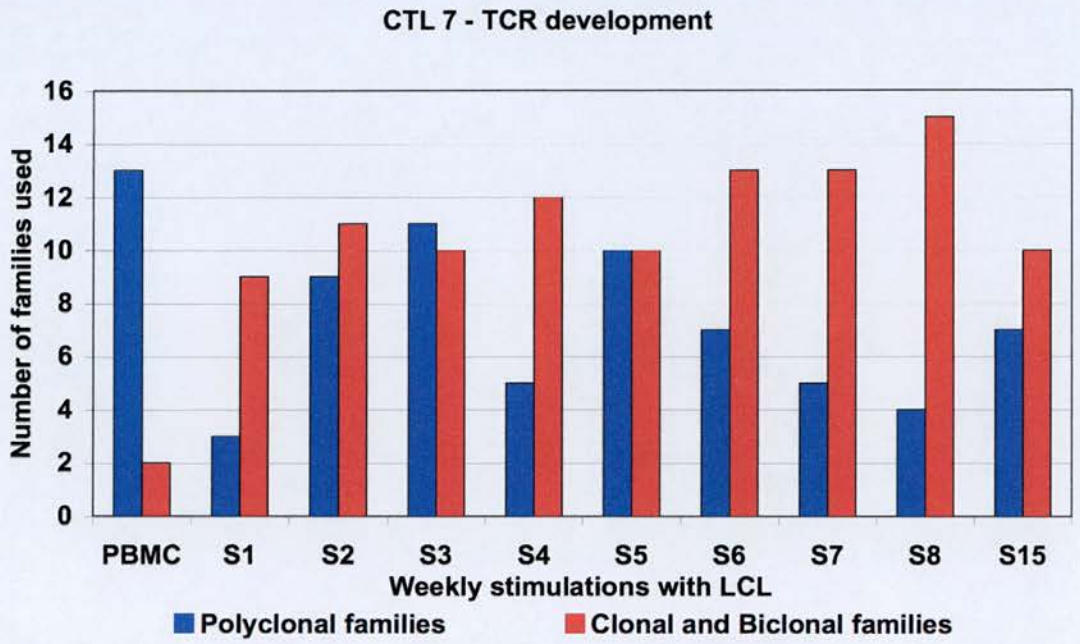


Figure 4.4: Development of TCR Vβ families in CTL 7.

4.3.2.1 Evolution of polyclonal families

The number of polyclonal families being used by each CTL changed from the first weekly stimulation analysed in phase 1 of growth (S1 to S4) to the last stimulation analysed in phase 2 of growth (S5-onwards). In CTLs 2, 3, 9 and 13 there was an increase in the number of polyclonal families in use over the course of each CTL's culture period, and there was a decrease in the number of polyclonal families in use in CTLs 4, 5 and 12. There was no change in the number of polyclonal families used by CTL 7 at the first stimulation and at the last stimulation analysed. With the exception of CTL 12, where the number of polyclonal families used decreased

continuously during the course of culture, the number of polyclonal families used by all other CTLs fluctuated over time.

Further analysis of results was carried out to determine whether there were particular families that remained polyclonal throughout the CTL culture period. This analysis is summarised in table 4.4. This table shows that the family Vβ-3 remained polyclonal in six of the CTLs investigated. This family was also in use in CTLs 7 and 13 but it was not polyclonal at all stimulations analysed. The family Vβ-12 remained polyclonal in five of the CTLs tested.

CTLs	**2**	**3**	**4**	**5**	**9**	**12**	**13**
Vβ families	2, 3, 12, 20, 22	2, 3, 5, 9, 12	3, 4, 12	3	3	3, 12	12

Table 4.4: Persistently polyclonal families in CTLs.

The Vβ-3 and Vβ-12 families were two of the most frequently used families by all of the CTLs during their course of culture (table 4.3). The Vβ-3 family was in use in all CTLs at all stimulations tested and the Vβ-12 family was in use at all stimulations tested in five CTLs (see table 4.3). Two of the most frequently used families therefore appear to remain polyclonal.

4.3.2.2 Evolution of clonal and biclonal families

In five CTLs – CTLs 3, 5, 7, 9 and 12 – there was an increase in the number of clonal and biclonal families from the first weekly stimulation with LCL analysed in phase 1 of growth (S1 to S4) to the last stimulation analysed in phase 2 of growth (S5-onwards). This increase was only continuous over time in CTLs 5 and 12. In the other four CTLs the number of clonal and biclonal families was variable over time. Further analysis of results was carried out to determine whether the increase in clonal and biclonal families could be attributed to particular families persisting over time becoming and/or remaining clonal or biclonal. There was no persistence of particular

Vβ families in CTLs 3 and 7, however families were found to persist in CTLs 4, 5, 9 and 12. These results are shown in table 4.5 a) to d).

a) CTL 4	PBMC	S4	S5	S6	S7	S8	S9
Clonal/Biclonal			21	21	21	21	21
Polyclonal	21	21					

b) CTL 5	PBMC	S1	S2	S3	S4	S5	S6
Clonal/Biclonal						17	17
Polyclonal	17	17	17	17	17		

c) CTL 9	PBMC	S1	S2	S3	S4	S5	S6
Clonal/Biclonal	12, 22		12	12	12	12, 22	12, 22
Polyclonal		12*	22	22	22		

* Vβ12 was polyclonal at S1 only.

d) CTL 12	S2	S3	S4	S5	S6
Clonal/Biclonal		4, 14, 16	4, 14, 16	2, 4, 14, 16	2, 4, 14, 16
Polyclonal	2, 4, 14, 16	2	2		

Table 4.5: Persistent Vβ families in CTLs a) 4, b) 5, c) 9 and d) 12.

These tables show that there was not one particular Vβ family that preferentially became and remained clonal or biclonal during the course of culture of the CTLs. Tables 4.5 a) and b) also show that in CTLs 4 and 5 respectively only one Vβ family remained clonal/biclonal during the course of culture; tables 4.5 c) and d) respectively show that in CTL 9 two Vβ families remained clonal/biclonal during the course of culture and in CTL 12 four Vβ families remained clonal/biclonal. This accounts only for a small portion of the clonal and biclonal families used at any one stimulation by the CTLs. The progression to clonality/biclonality is therefore variable in terms of which families become clonal/biclonal following stimulation

with LCL but it is also "reversible" as families were found to be clonal/biclonal at one stimulation and polyclonal at the next stimulation.

The evolution of two persistent clonal/biclonal Vβ families in two different CTLs is represented in figures 4.5 and 4.6. Family Vβ-17 from CTL 5 is shown in figure 4.5 and the family Vβ-22 from CTL 9 is shown in figure 4.6. Each bar represents one clone.

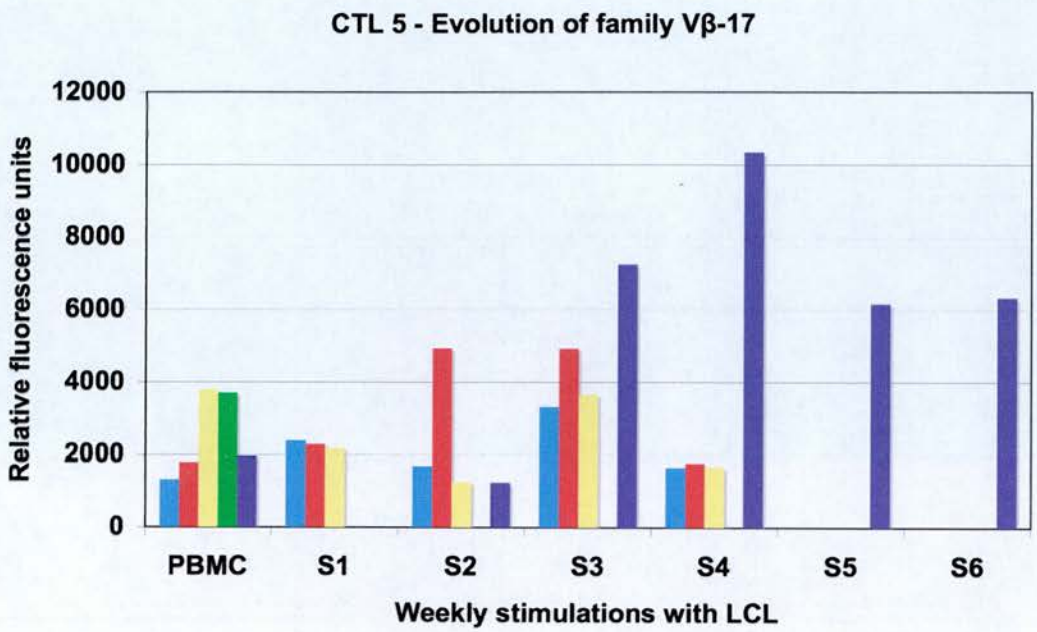


Figure 4.5: Evolution of family Vβ-17 in CTL 5. Each bar represents one clone.

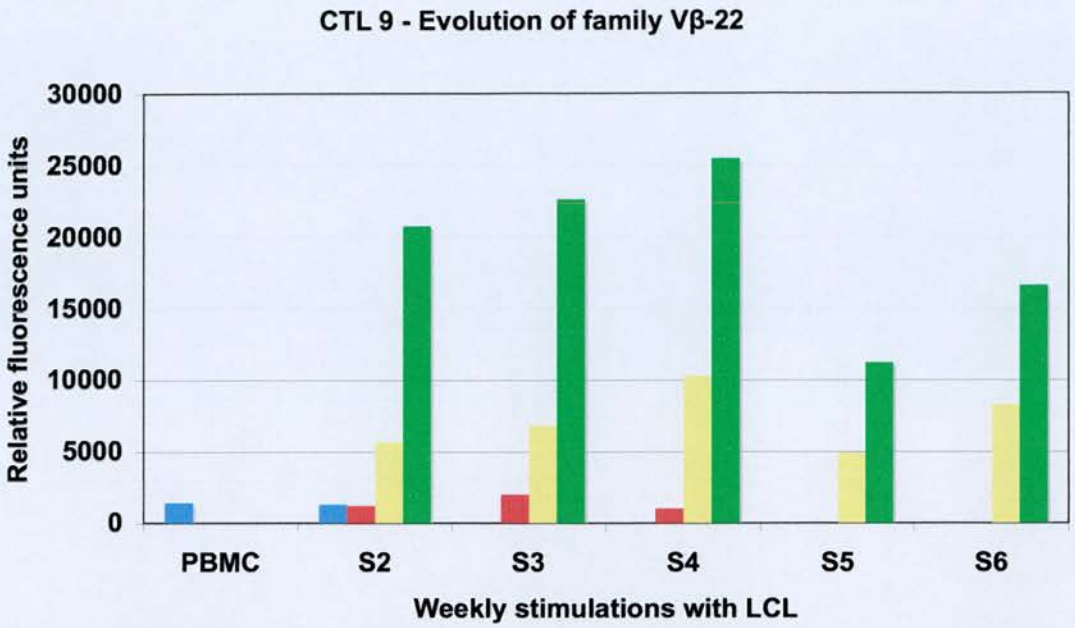


Figure 4.6: Evolution of family Vβ-22 in CTL 9. Each bar represents one clone.

4.3.2.3 Summary

In four CTLs (4, 5, 7 and 12) the number of polyclonal families used decreased and the number of clonal/biclonal families increased during the period of culture, resulting in there being more clonal/biclonal families than polyclonal families at the last stimulation tested for each CTL. In two CTLs (2 and 13) the number of polyclonal families increased and the number of clonal/biclonal families decreased resulting in there being more polyclonal families than clonal/biclonal families at the last stimulation tested for each CTL. In CTL 3 both the number of polyclonal and clonal/biclonal families increased, resulting in there being more polyclonal families than clonal/biclonal families at the last stimulation tested. In CTL 9, the number of polyclonal families increased and the number of clonal/biclonal families was the same in the PBMC than at S6, but there remained more clonal/biclonal families than polyclonal families.

Stimulation with LCL appeared to favour the use of family Vβ-3, which was used by all CTLs at all stimulation tested. The use of family Vβ-3 was preferentially

polyclonal as found in six CTLs. Interestingly, family Vβ-12, which was found to be preferentially polyclonal over time in five CTLs, was found to have become and remain clonal/biclonal in another CTL. Stimulation of the CTLs using autologous LCL did not appear to drive preferential clonal/biclonal use of particular Vβ families in the CTLs over time.

Based on these results it appears that development of the Vβ families is a fluid and dynamic process where families may be in use at one stimulation but not at the next. Furthermore, these families may be polyclonal or clonal/biclonal families at one stimulation and the converse at the next stimulation.

4.4 Development of T-cell clones within Vβ families

The development of T-cell clones within Vβ families was studied next, in order to determine whether the dynamic and fluid development of Vβ families was associated with the presence, absence or persistence of certain T-cell clones during culture.

4.4.1 Investigation of T-cell clones within clonal/biclonal families

The families that became clonal/biclonal were Vβ-2, Vβ-4, Vβ-12, Vβ-14, Vβ-16, Vβ-17, Vβ-21 and Vβ-22. These are listed in table 4.5 along with the CTLs in which they were identified. The development of two of these clonal/biclonal families is represented in figures 4.7 and 4.8.

Figure 4.7 shows that family Vβ-17 was polyclonal for the first four stimulations, although by S3, a dominant T-cell clone with a TCR CDR3 length of 239bp emerged in CTL 5. This clone persisted over time, whilst the other TCR clones became less prevalent and ultimately disappeared at S5.

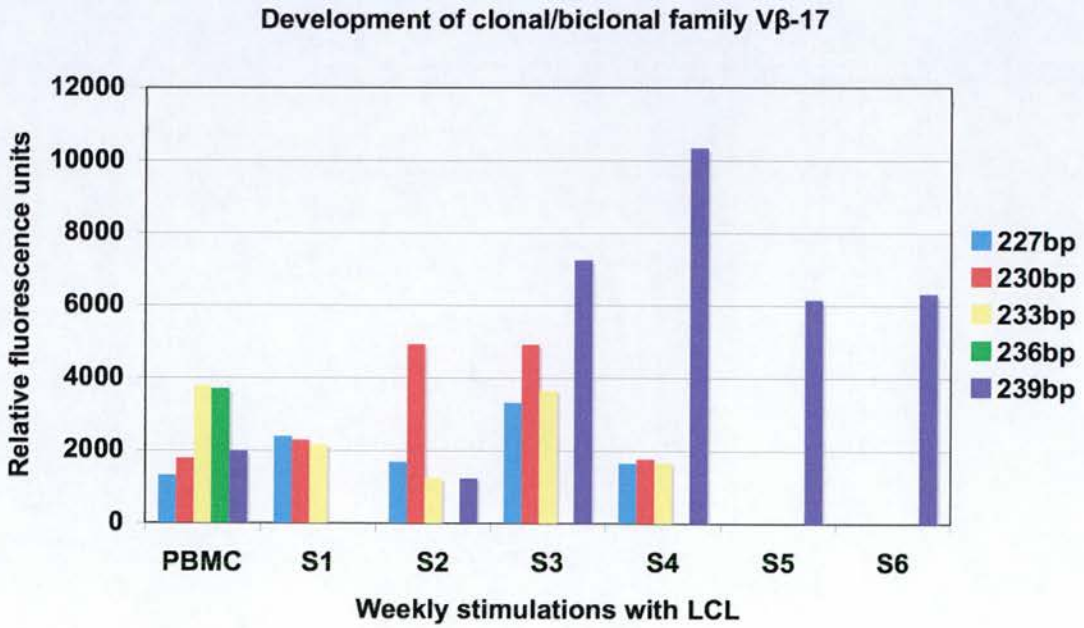


Figure 4.7: Development of clonal/biclonal family Vβ-17 in CTL 5.

Figure 4.8 shows that at S1 there were three T-cell clones with different TCR CDR3 length, with one dominant clone with a CDR3 of 280bp in CTL 9. This dominant clone persisted throughout the period of culture, whilst the two other clones disappeared. The clonality of family Vβ-12 was due to the persistence one particular clone over time (from S1).

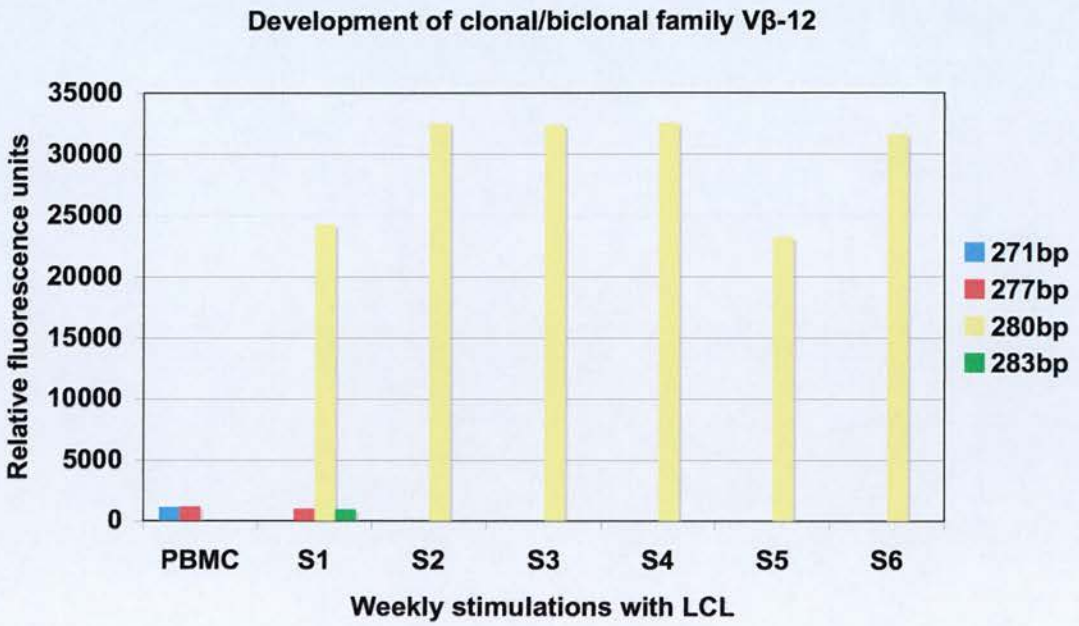


Figure 4.8: Development of clonal/biclonal family Vβ-12 in CTL 9.

The development of the other Vβ families, which became clonal/biclonal is summarised in tables 4.6 a) to f). The columns highlighted in blue show which clones persisted from the earliest stimulation analysed to the last stimulation analysed.

a) Vβ-2 – CTL 12	Relative expression of TCR clones in rfu			
CDR3 length	**196bp**	**199bp**	**202bp**	**205bp**
S2	14720 rfu	11147 rfu	16362 rfu	5627 rfu
S4	9988 rfu	6844 rfu	6049 rfu	3927 rfu
S5	10960 rfu	5713 rfu	6040 rfu	1127 rfu
S6	1708 rfu	2015 rfu		
S7	3749 rfu			1227 rfu

b) Vβ-4 – CTL 12	Relative expression of TCR clones in rfu				
CDR3 length	**334bp**	**337bp**	**340bp**	**343bp**	**346bp**
S2	2535 rfu	2373 rfu	5621 rfu	30883 rfu	5416 rfu
S4		1070 rfu	2193 rfu	22806 rfu	
S5				16290 rfu	
S6				4095 rfu	
S7				11260 rfu	

c) Vβ-14 – CTL 12	Relative expression of TCR clones in rfu			
CDR3 length	**373bp**	**376bp**	**379bp**	**380bp**
S2	1688 rfu	2592 rfu	32670 rfu	
S4		1164 rfu	24530 rfu	
S5			17169 rfu	17897 rfu
S6			6800 rfu	
S7			5972 rfu	

d) Vβ-16 – CTL 12	Relative expression of TCR clones in rfu			
CDR3 length	**242bp**	**245bp**	**248bp**	**254bp**
S2	2580 rfu	1768 rfu	2046 rfu	32427 rfu
S4	1429 rfu			32647 rfu
S5				32760 rfu
S6				1200 rfu
S7				14863 rfu

e) Vβ-21 – CTL 4	Relative expression of TCR clones in rfu					
CDR3 length	**184bp**	**187bp**	**190bp**	**193bp**	**196bp**	**199bp**
PBMC	5862 rfu	4120 rfu	6326 rfu	4055 rfu	3331 rfu	3035 rfu
S4		1201 rfu		9567 rfu	2260 rfu	
S5				17339 rfu		1268 rfu
S6				7606 rfu		
S7				23889 rfu	1054 rfu	
S8				7751 rfu		
S9				2663 rfu		

f) Vβ-22 – CTL 9	Relative expression of TCR clones in rfu			
CDR3 length	**238bp**	**241bp**	**244bp**	**253bp**
PBMC	1457 rfu			
S2	1328 rfu	1245 rfu	5709 rfu	20768 rfu
S3	2016 rfu	6863 rfu	22640 rfu	
S4	1049 rfu	10315 rfu	25508 rfu	
S5			4954 rfu	11230 rfu
S6			8312 rfu	16621 rfu

Table 4.6: Development of clonal/biclonal family a) Vβ-2, b) Vβ-4, c) Vβ-14, d) Vβ-16, e) Vβ-21 and f) Vβ-22. Relative expression of peaks is expressed in relative fluorescence units (RFU).

One T-cell clone was found to persist in culture for families Vβ-2, Vβ-4, Vβ-14, Vβ-16 and Vβ-17. One clone (244bp) persisted for family Vβ-22 and family Vβ-12 (280bp) from the S2 but not from S0 (PBMC). The clonality of five out of eight clonal/biclonal families (Vβ-2, Vβ-4, Vβ-14, Vβ-16 and Vβ-21) was attributable to the persistence on one T-cell clone throughout the period of culture. In families Vβ-17 and Vβ-22, the clones found at the last stimulation analysed were not detected throughout the period of culture analysed.

4.4.2 Investigation of T-cell clones within polyclonal families

The Vβ families which remained polyclonal throughout the culture of the CTLs, were investigated in order to determine whether the same T-cell clones persisted over time. The Vβ families which were investigated are given in table 4.4.

4.4.2.1 Polyclonal families Vβ-2, Vβ-4, Vβ-5, Vβ-9, Vβ-20 and Vβ-22

In CTL 2 family Vβ-2, there were four clones, which were present at S2 through to S5. In family Vβ-22, there were four clones at S2 and six at S5, four of which were also present as at S2. For family Vβ-20, there were four clones at S2 and three at S5, two of which were the same as at S2.

In CTL 3 family Vβ-5, there were six clones at S3 and three at S5, all three of which had also been found at S3. In family Vβ-9, there were three clones at S3 and three at S5 but only one of those clones had been found at S3.

In CTL 5 family Vβ-4, there were five clones in the PBMC (four at S4) and three at S9; all three of these clones were found throughout the period of culture.

Based on the data gathered for CTLs 2, 3 and 5 T-cell clone persistence also occurs in polyclonal families but the number of clones found in a family can vary during culture.

4.4.2.2 Polyclonal families Vβ-3 and Vβ-12

As family Vβ-3 was found to be polyclonal at all stimulations in six CTLs, the persistence of T-cell clones was investigated within the CTLs but also between the CTLs. The same was done for family Vβ-12 as it was detected in five of the CTLs.

a) Family Vβ-3

Between three and six clones were detected at the earliest stimulation analysed. The size of the CDR3 lengths of these T-cell clones were: 192bp, 195bp, 198bp, 201bp, 204bp and 207bp. Between two and five of these clones were detected at the latest stimulation analysed in the CTLs. However, the T-cell clone with the CDR3 length of 204bp was found in all CTLs tested at all stimulations tested.

b) Family Vβ-12

Between six and eight clones were detected at the earliest stimulation analysed. The CDR3 lengths of these T-cells clones were: 267bp, 270bp, 273bp, 276bp, 279bp, 282bp, 285bp and 288bp. No single clone was detected in all CTLs, but three clones (273bp, 276bp and 279bp) were detected at all stimulations in four CTLs. The polyclonality of the Vβ-12 family was not centred around one clone, as with family Vβ-3, but three clones.

4.4.3 Summary

The families that remained or became clonal/biclonal during the course of culture of the CTLs investigated were all different. The clonality/biclonality of six Vβ families was attributable to the presence of one clone from the earliest stimulation analysed (S1 to S4) to the last stimulation analysed (S5 onwards). In two Vβ families, the T-cell clone (s) that conferred the family its clonality/biclonality was not present throughout the period of culture.

There were eight polyclonal families, which were detected throughout the course of culture in seven CTLs. Family Vβ-3 was detected in all CTLs and family Vβ-12 was

detected in five of the CTLs. The other polyclonal families were found only once in four CTLs. In family Vβ-3, polyclonality in all CTLs revolved around one T-cell clone and in family Vβ-12, polyclonality in all CTLs revolved around three T-cell clones. This suggest a degree of conservation of CDRs in a polyclonal setting. It also suggests that LCLs present antigen (s) of a similar nature to CTLs in order for these particular clones to persist in CTLs generated from PBMC of donors with different HLA types.

4.5 Discussion

Memory T-cells to common pathogens (CD62L negative), such as HCMV, display a restricted TCR repertoire in terms of Vβ family clonality, which may be associated with chronic antigen exposure (Foster *et al*, 2004). The CTLs analysed in this project were stimulated weekly with LCLs, providing the CTLs with chronic antigen exposure. However, the CTLs analysed demonstrate that at the level of Vβ family usage and also at the level of the clonality of the Vβ families there was no restriction in the TCR repertoire. There was usage of almost all Vβ families and there was no discernable pattern in terms of specific Vβ families becoming clonal/biclonal during the course of culture. This type of TCR repertoire has been termed "private" with considerable inter-CTL (hence PBMC donor) diversity and variation, making each CTL unique. There was, however, the persistence of one clone from family Vβ-3, which was present in all CTLs at all stimulations analysed. This would be considered a "public" clonotype, which is not specfic to one CTL (hence PBMC donor). "Public" clonotypes such as the one found in family Vβ-3, have been found in HLA-A2 individuals reactive to the EBV lytic antigen BMLF-1 (Lim *et al*, 2000). In this setting, the detection of this clonotype was not restricted to a specific HLA type.

Taken together, these results indicate that weekly LCL stimulation maintained a broad and diverse TCR repertoire in the CTLs. The presence of antigen/MHC class I complexes with prominent features on the LCLs may be responsible for this, as it has been shown that featureless antigen/MHC class I complexes reduced the diversity of TCR usage (Turner *et al*, 2005). Additionally, this diversity and breadth of the TCR repertoire may have also arisen because the contraction of initially expanded cells following primary antigen stimulation was prevented by the addition of IL-2 to the CTLs.

Such breadth and diversity in the TCR repertoire may be essential in the context of PTLD treatment with allogeneic CTLs, along with the potential structural flexiblity of the CDR3 of the TCR (Garcia *et al*, 1998), as it may increase the likelihood of

CTL recognition of antigen presented particularly in a partially HLA-matched context.

Antigen recognition by the TCR is the key to triggering the effector mechanisms of the CTLs. Having established that there was no distinct pattern in the development of the TCR but that its stimulation did induce phenotypic changes, consistent with an antigen-experienced/mature phenotype, the function of the CTLs was investigated next.

Chapter 5

Cytotoxic pathways and cytolytic proteins of the CTLs

5.1 Introduction

5.2 Cytotoxicity assays of the CTLs

5.3 Detection of mRNA transcripts for perforin, granzyme B, granulysin and FasL in the CTLs

5.4 Expression of cytolytic proteins in the CTLs

5.5 Discussion

5.1 Introduction

Investigation into the cytotoxic pathways of the CTLs was carried out on three of the CTLs (5, 7 and 9) described in previous chapters. There were not enough stored cells from the other CTLs to carry out further investigations. In order to complement these results, 9 other CTLs (15-24) were also investigated. These CTLs were in phase 2 of growth. The cytolytic proteins and the cytotoxic pathway(s) employed by these CTLs *in vitro* had not been previously established. Additionally, it was established in chapter 3 that CTLs progressed towards a highly differentiated antigen-experienced phenotype that has been associated with the acquisition of cytolytic molecules such as perforin in *ex vivo* CTLs (Appay *et al*, 2002a; Appay *et al*, 2002b). The cytolytic pathways and cytolytic proteins of the CTLs were therefore investigated next.

The cytolytic proteins investigated were perforin, granzyme B, FasL and granulysin, which have all been found in both NK cells and cytotoxic T-cells (Lieberman, 2003). However these cytotoxic T-cells were not generated using the method used in this work and described by Haque *et al* (2001) and Wilkie *et al* (2004). Perforin is synthesised as a 70kDa inactive pro-protein that undergoes cleavage at the C-terminus resulting in the 60kDa active cytolytic form of the protein, which is stored in cytolytic granules (Uellner *et al*, 1997). Perforin bears a degree of homology to the C9 component of the complement pathway and it has the ability to penetrate membrane lipid bilayer and polymerise leading to pore formation. Granzyme B is a 32kDa serine protease (Trapani and Sutton, 2003), which is found, in conjunction with perforin, in cytolytic granules. Granzyme B can induce apoptosis by cleavage of pro-caspase 3, as well as by causing outer mitochondrial membrane damage triggering the caspase cascade and leading to the release of pro-apoptotic factors, all of which ultimately lead to DNA fragmentation and target cell apoptosis (Lieberman, 2003). Fas ligand (FasL, also known as CD95L) is a type II transmembrane protein typically expressed at the surface of cytotoxic T-cells and NK cells. FasL is a member of the tumour necrosis factor family that binds to Fas (also known as CD95). The binding of FasL to Fas induces a series of events that leads to the

activation of caspase 8 and in turn of caspase 3 culminating in apoptosis of the target cell (Aggarwal, 2003). Granulysin is a 9kDa saposin-like protein, which is found in NK cells and cytotoxic T-cells (Pena *et al*, 1997). It is synthesised as a 15kDa inactive precursor, which is post-translationally cleaved into the active 9kDa from and it is found in cytolytic granules (Hanson *et al*, 1999). Apoptosis results from granulysin binding to the target cell membrane, leading to a disruption in the flux of calcium and potassium ions as well as the generation of ceramide, both of which cause mitochondrial membrane damage and ultimately lead to the activation of caspase 3 resulting in apoptosis of the target cell (Kaspar *et al*, 2001; Krensky and Clayberger, 2005).

The main pathways by which the cytotoxic T-cells may effect their cytotoxic role were investigated in the first instance, followed by the expression of the cytolytic protein mRNA and finally the expression of the proteins themselves.

5.2 Cytotoxicity assays of CTLs

The cytotoxicity of the CTLs in phase 2 of growth was assessed using a standard 4-hour chromium release assay as described in chapter 2 section 2.2. The targets used in these assays were autologous LCLs, HLA-mismatched LCLs and NK-cell sensitive K562 cells. A modified 4-hour chromium release assay was used to determine the nature of the cytotoxic pathways used by the cells within the CTLs. Autologous LCLs were used as the sole target in the modified 4-hour chromium release assay, which was carried out as described in chapter 2 section 2.2.

5.2.1 Standard 4-hour chromium release cytotoxicity assay

The results of the standard 4-hour chromium release cytotoxicity assays carried out are given in table 5.1. Table 5.1 gives the percentage of target cell lysis by each of the CTL at effector cell:target cell (E:T) ratios of 20:1, 10:1 and 5:1.

CTLs	E:T ratio – 20:1			E:T ratio – 10:1			E:T ratio – 5:1		
	Auto LCL	Mis LCL	K562	Auto LCL	Mis LCL	K562	Auto LCL	Mis LCL	K562
5	65.4%	16.8%	1.7%	58.8%	11.3%	0.5%	51.9%	6.8%	0%
7	49.0%	2.4%	0%	27.1%	2.4%	0.3%	31.2%	0%	0%
9	55.5%	15.9%	5.4%	49.1%	11.8%	3.9%	37.7%	8.6%	4.1%
15	36.7%	0.9%	1%	33.9%	0%	0.5%	30.6%	0%	0%
16	27.7%	0.4%	2.8%	29.5%	0.5%	1.1%	22.8%	0.6%	0.3%
17	43%	2.8%	3.1%	41.2%	0.8%	0.6%	33.7%	0%	0%
19	79.5%	0.3%	0%	77.9%	62.4%	1.3%	86.1%	40.7%	0%
20	47.2%	3.8%	5.8%	48.8%	0%	3.6%	48.6%	0%	2%
21	23.8%	0.1%	2.7%	16.2%	2%	1.6%	14.4%	0.9%	0.5%
22	23.7%	5.7%	5.6%	19%	13%	1.3%	15.2%	12.6%	2.1%
23	38.6%	16.4%	11%	34.2%	17.6%	7%	21.3%	10.8%	2.3%
24	34%	10%	1.3%	29%	9%	0.5%	20%	5%	1.5%

Table 5.1: Standard 4-hour chromium release cytotoxicity assays results.

Figure 5.1 represents the results of the cytotoxicity assay results given in table 5.1 at an E:T ratio of 20:1.

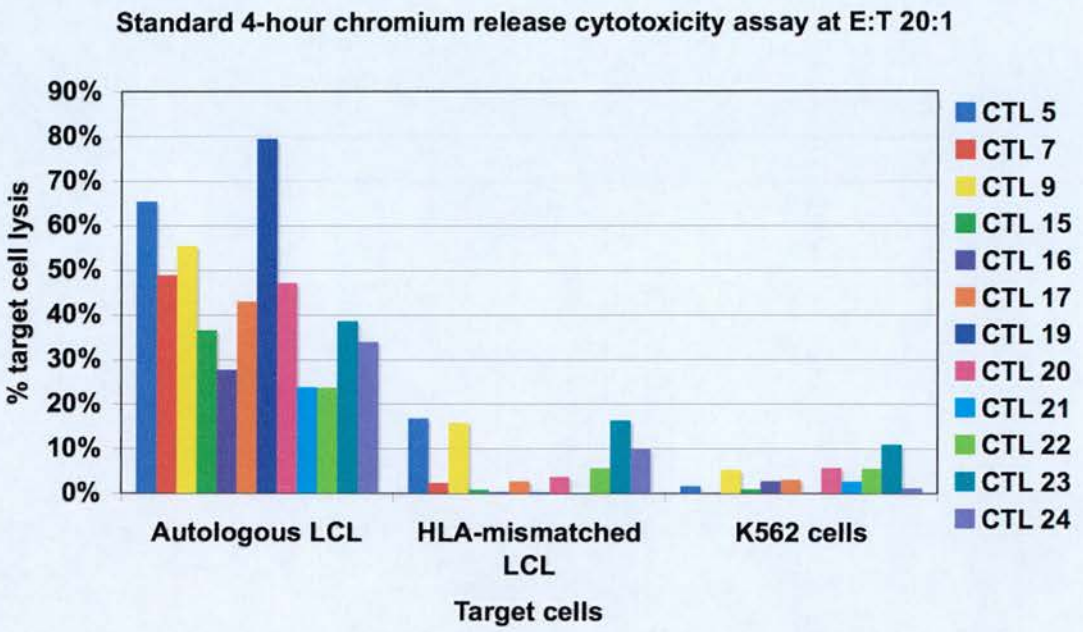


Figure 5.1: Standard 4-hour chromium release cytotoxicity assay at E:T 20:1.

Figure 5.2 is graphical representation of full cytotoxicity results for one CTL (CTL 9) at E:T ratios of 20:1, 10:1 and 5:1.

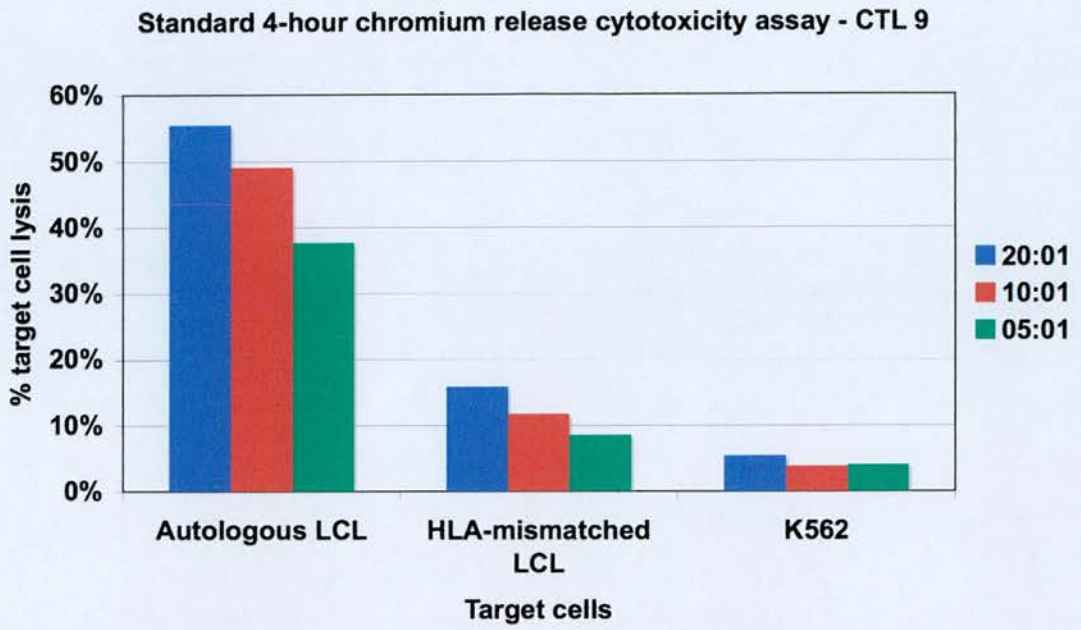


Figure 5.2: Standard 4-hour chromium release cytotoxicity assay for CTL 9 at E:T ratios of 20:1, 10:1 and 5:1.

The cytotoxicity results demonstrate that all of the CTLs exhibit greatest lysis towards their autologous LCL, which is indicative of target specificity. Hence, the cytotoxic pathway(s) employed by these CTLs were investigated using the modified 4-hour chromium release cytotoxicity assay described below.

5.2.2 Modified 4-hour chromium release cytotoxicity assay

A modified 4-hour chromium release assay using concanamycin A (CMA), brefeldin A (BFA) and ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) was set up to identify the potential pathways by which the cytotoxic T-cells in the CTLs effect their cytotoxic role. CMA prevents the acidification of vacuoles containing perforin, which leads to their degradation (Kataoka *et al*, 1996; Kataoka *et al*, 1994). BFA inhibits Golgi-mediated transport (Zenewicz *et al*, 2004). The last inhibitor used was EGTA, which chelates free extracellular calcium that is necessary for the exocytosis of cytolytic granule-containing vacuoles (Sun *et al*, 2002). This modified 4-hour chromium release assay was carried out as described in section 2.2.

5.2.2.1 Modified 4-hour chromium release cytotoxicity assay results

The results of this modified 4-hour chromium release cytotoxicity assay are shown in figure 5.3 (E:T 20:1). Figure 5.3 is a scatter diagram, where the bold line represents the median of each data set. The results of the modified 4-hour chromium release cytotoxicity assay used to generate figure 5.3 are shown in table A5.1 in appendix A5.

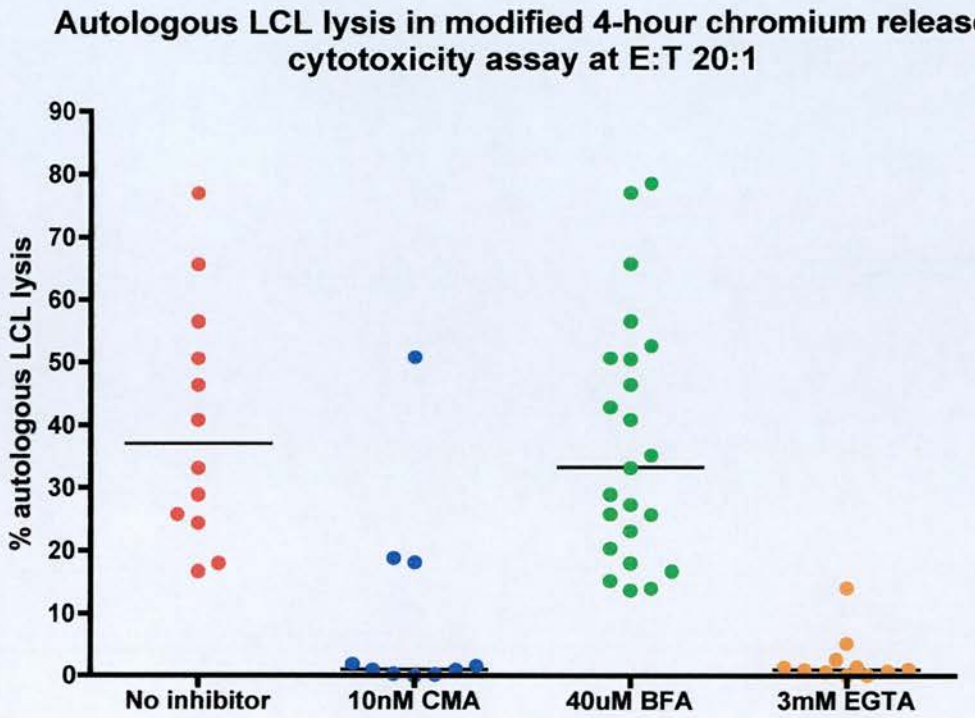


Figure 5.3: Autologous LCL lysis in modified 4-hour chromium release cytotoxicity assay (E:T 20:1). n=12 CTLs.

This modified 4-hour chromium release cytotoxicity assay was also carried out at E:T 10:1 and 5:1 and the results are shown in figure 5.4 and figure 5.5 respectively, where the bold line represents the median of each data set. The tables of results for E:T 10:1 and 5:1 are in appendix A5 (table A5.2 and A5.3).

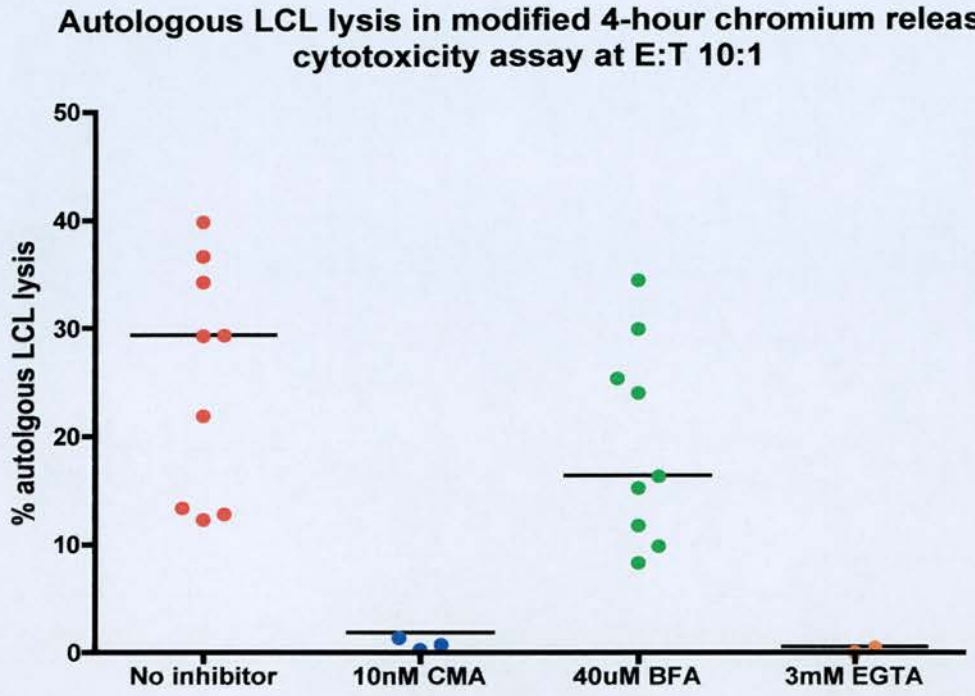


Figure 5.4: Autologous LCL lysis in modified 4-hour chromium release cytotoxicity assay (E:T 10:1). n=9 CTLs.

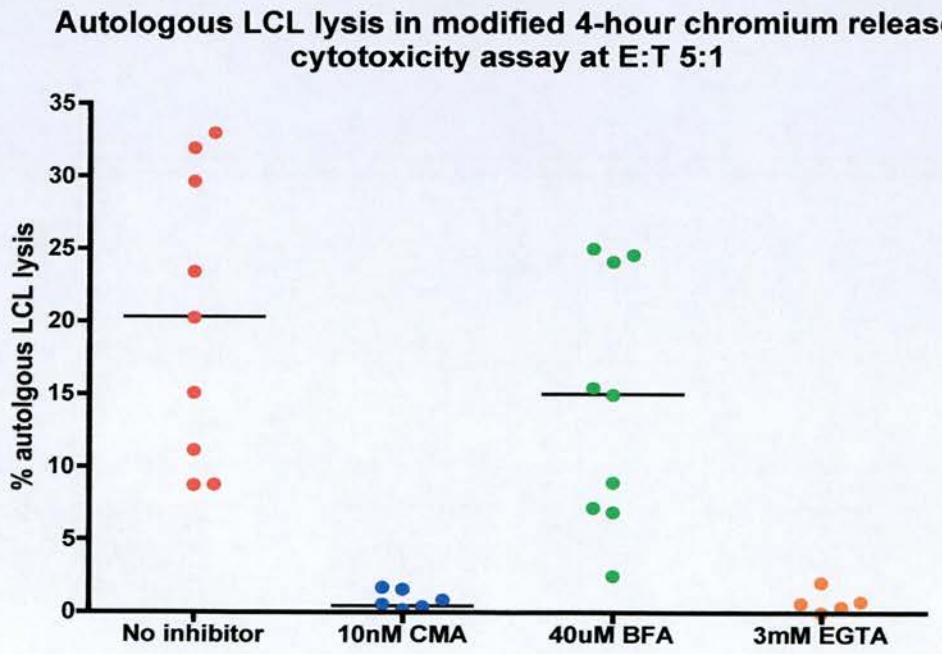


Figure 5.5: Autologous LCL lysis in modified 4-hour chromium release cytotoxicity assay (E:T 5:1). n=9 CTLs.

5.2.2.2 Result analysis

The Mann-Whitney test was used to analyse the results of the modified 4-hour chromium release cytotoxicity assays at E:T 20:1, 10:1 and 5:1. The results of the Mann-Whitney tests carried out are summarised in table 5.2.

% autologous LCL lysis	E:T 20:1	E:T 10:1	E:T 5:1
No inhibitor vs 10nM CMA	$p = 0.0007$*	$p < 0.0001$*	$p < 0.0001$*
No inhibitor vs 40μM BFA	$p = 0.2855$	$p = 0.2581$	$p = 0.2581$
No inhibitor vs 3mM EGTA	$p < 0.0001$*	$p < 0.0001$*	$p < 0.0001$*

Table 5.2: Results of Mann-Whitney tests on modified 4-hour chromium release cytotoxicity assays at E:T 20:1, 10:1 and 5:1. * significant at $p < 0.05$.

These results show that both CMA and EGTA significantly inhibited autologous LCL lysis in comparison to the control without inhibitor, whereas BFA did not. This indicates that the principal cytotoxic pathway in the CTLs tested is mediated by calcium-mediated granule-exocytosis and not by Golgi-mediated transport of cytolytic proteins.

5.2.2.3 Reduction in cytotoxicity in the presence of inhibitors

The percentage reduction in cytotoxicity achieved by the inhibitors was determined using the results of the modified 4-hour chromium release cytotoxicity assays given in figures 5.3, 5.4 and 5.5. The results are given in table 5.3 for E:T 20:1. The mean and median percentage reduction in cytotoxicity induced by the inhibitors at E:T 20:1 remained similar at E:T 10:1 and 5:1, as shown in table 5.4. The full tables of results for percentage reduction in cytotoxicity induced by the inhibitors at E:T 10:1 and 5:1 are given in appendix A5 (tables A5.4 and A5.5).

	% reduction in autologous LCL lysis at E:T 20:1		
	Induced by CMA	**Induced by BFA**	**Induced by EGTA**
CTL 5	71.2%	0%	98.4%
CTL 7	34.1%	34.4%	81.7%
CTL 9	67.7%	7.0%	90.7%
CTL 15	96.2%	20.4%	94.4%
CTL 16	96.4%	24.1%	97.5%
CTL 17	99.1%	15.6%	99.6%
CTL 19	99.8%	33.0%	99.7%
CTL 20	100%	30.3%	100%
CTL 21	100%	0.6%	96.7%
CTL 22	89.1%	15.8%	95.8%
CTL 23	93.6%	16.3%	91.0%
CTL 24	98.6%	43.7%	89.0%
Mean % reduction in cytotoxicity	87.1%	20.1%	94.5%
Median % reduction in cytotoxicity	96.3%	18.3%	96.2%

Table 5.3: Percentage reduction in autologous LCL lysis induced by CMA, BFA and EGTA at E:T 20:1. n=12 CTLs.

	Induced by CMA	Induced by BFA	Induced by EGTA
Mean % reduction in cytotoxicity at 10:1	97.1%	19.4%	99.4%
Median % reduction in cytotoxicity at 10:1	100%	18.4%	100%
Mean % reduction in cytotoxicity at 5:1	96.7%	24.4%	98.5%
Median % reduction in cytotoxicity at 5:1	97.3%	21.1%	100%

Table 5.4: Mean and median percentage reduction in autologous LCL lysis induced by CMA, BFA and EGTA at E:T 10:1 and 5:1. n=9 CTLs.

These results support the results of the Mann-Whitney tests shown in table 5.2, which indicate that both CMA and EGTA have significant inhibitory effect on the cytotoxicity of the CTLs whereas BFA does not. The potential cytolytic proteins involved in these pathways were investigated next.

5.3 Detection of mRNA transcripts for perforin, granzyme B, granulysin and FasL in the CTLs

5.3.1 Perforin, granzyme B, granulysin and FasL RT-PCR

As mentioned in chapter 2 section 2.3, the primers used to detect perforin, granzyme B, granulysin and FasL mRNA transcripts were designed using Primer 3 software. Cells expressing the CD56 cell-surface marker (an NK cell marker) were used as a positive control as cells expressing this marker have been shown to express perforin, granzyme B, granulysin and FasL (Lieberman, 2003). TK-143B cells (adherent human bone osteosarcoma cells) were used as a negative control. The TK-143B cells were growing in tissue culture and were therefore readily available. However, the CD56-expressing cells had to be positively selected from a CTL that had been previously found to be rich in CD56 cells. PBMCs from two healthy laboratory volunteers were also used a positive control as up to 10% of PBMC are CD56-expressing NK cells (Williams *et al*, 2005).

5.3.2 RT-PCR positive and negative controls

In addition to the PBMCs another positive control was generated using a CTL rich in CD56-expressing cells. 64% of the cells in that CTL expressed the CD56 marker as determined by FACS analysis. These CD56-expressing cells were enriched by EasySep positive selection (this was carried out as described in chapter 2 section 2.2). Following the positive selection, the CD56 enriched fraction was made up of 80% of cells expressing CD56 as shown in figure 5.6. Peaks in the area below the marker, shown in figure 5.6, were considered as positive (peaks left of the marker were considered as negative).

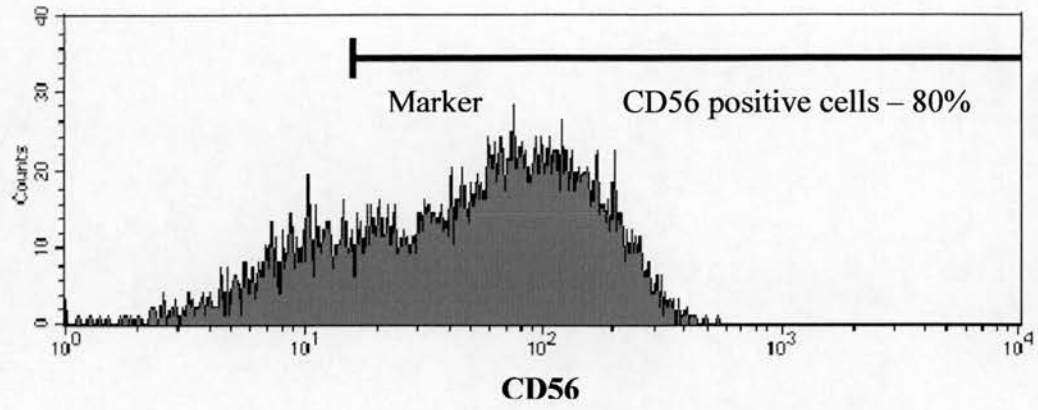


Figure 5.6: Enriched CD56 fraction following EasySep positive selection.

RNA extraction, cDNA synthesis and control β-actin RT-PCR were thereafter carried out on these cells. Figure 5.7 a) shows an agarose gel of β-actin RT-PCR using RNA extracted from CD56-expressing cells (two samples), which shows that the RNA was free of DNA contamination. Figure 5.7 b) shows an agarose gel of β-actin RT-PCR using cDNA synthesised from the RNA (extracted from CD56-expressing cells), which shows that the reaction was successful.

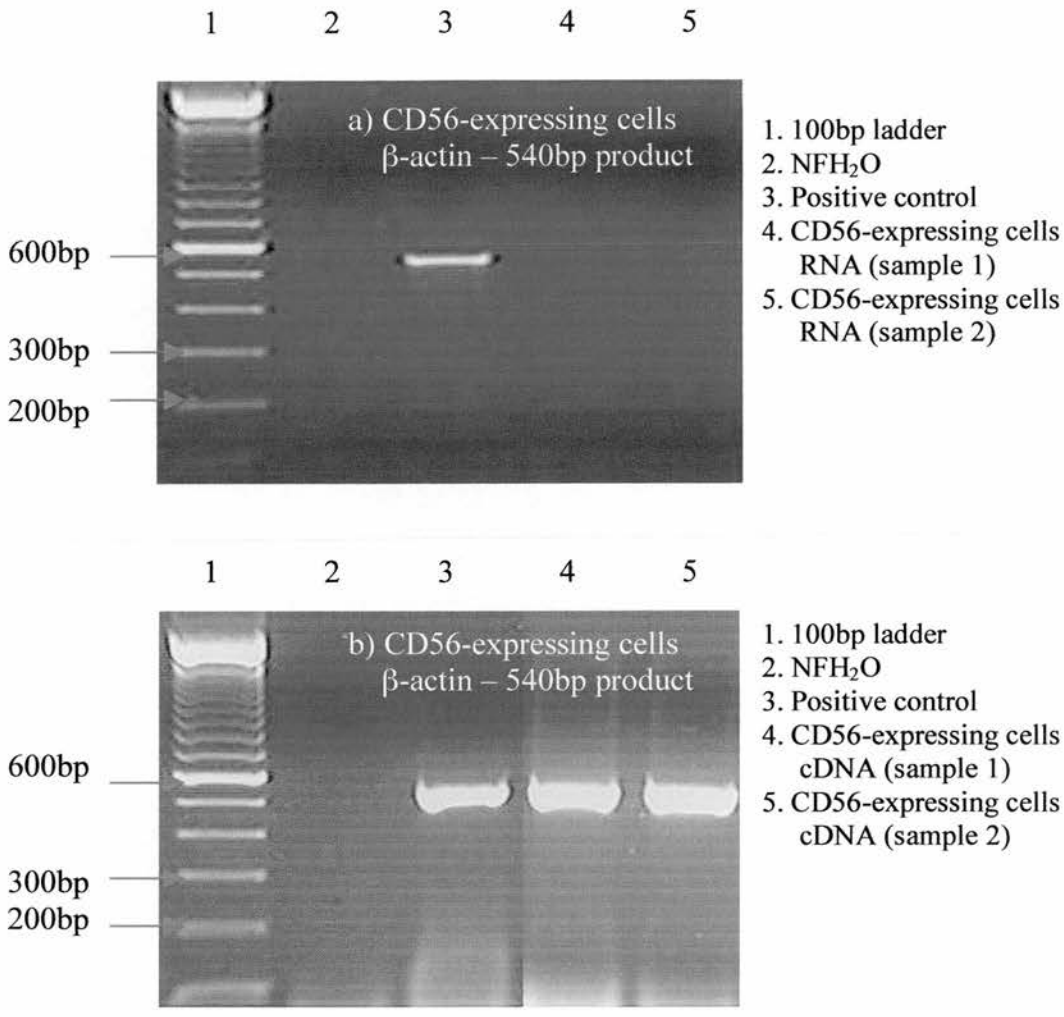


Figure 5.7: β-actin RT-PCR using a) RNA from CD56-expressing and b) cDNA from CD56-expressng cells.

Figure 5.8 a) to d) shows that PBMCs (from two healthy laboratory volunteers) expressed mRNA transcripts for perforin, granzyme B, granulysin and FasL and that CD56-expressing cells also expressed mRNA transcripts for granzyme B, granulysin and FasL, as RT-PCR products of the appropriate size can be seen on the agarose gels. CD56-expressing cells also expressed perforin mRNA transcripts (see figure 5.9 a)).

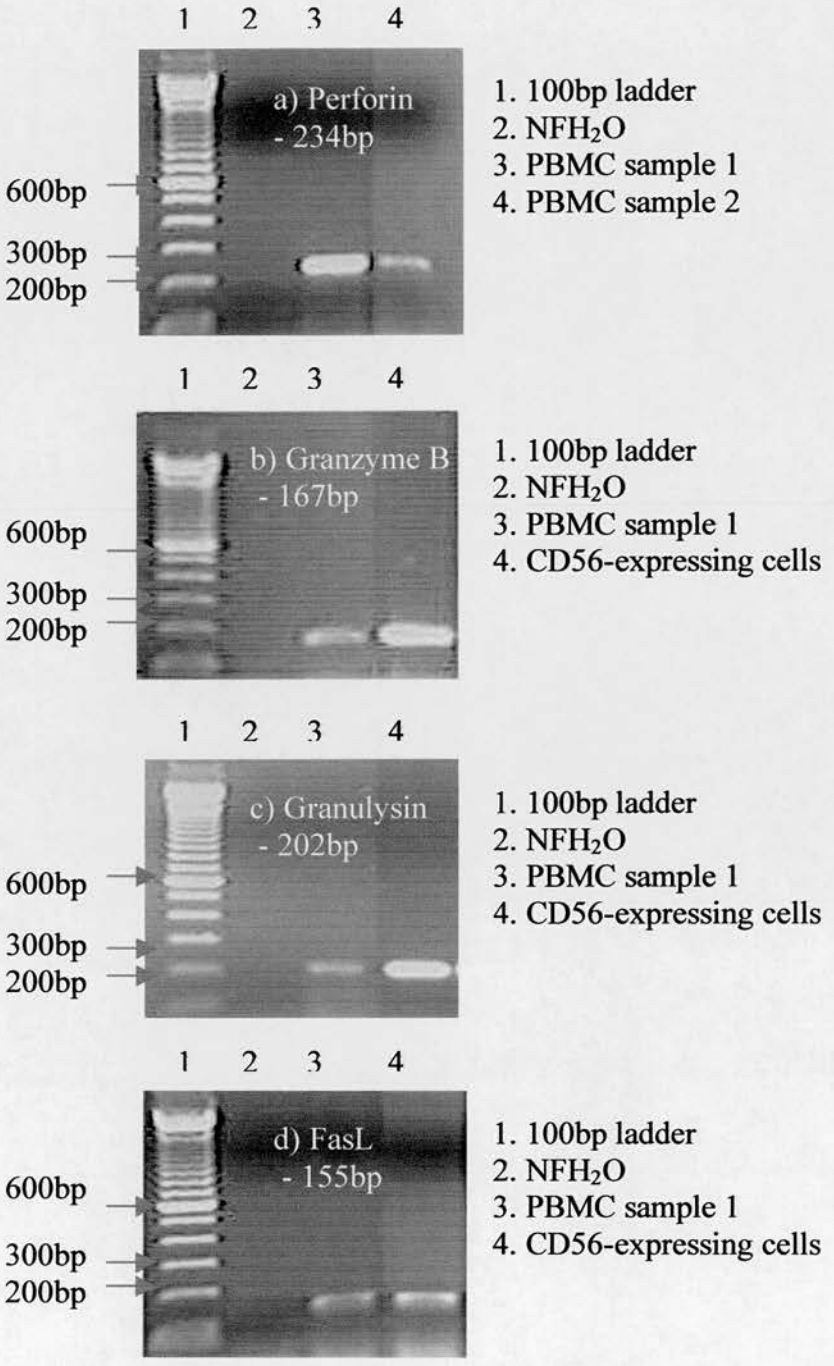


Figure 5.8: Agarose gel following a) perforin, b) granzyme B, c) granulysin and d) FasL RT-PCR.

5.3.3 Perforin, granzyme B, granulysin and FasL RT-PCR on CTLs

The RT-PCRs for perforin, granzyme B, granulysin and FasL were carried as previously described in chapter 2 section 2.3 and these were carried out on the CTLs and at all stimulations given in table 5.5, as well as on CTLs 15 to 24 which were in phase 2 of growth (at stimulations ranging from S7 to S16).

CTLs	Stimulations at which RT-PCR was carried out	
	Phase 1 of growth (S1-S4)	**Phase 2 of growth (S5-onwards)**
CTL 5	S1, S2, S3, S4	S5, S6
CTL 7	S1, S2, S3, S4	S5, S6, S7
CTL 9	S1, S2, S3, S4	S5, S6

Table 5.5: CTLs and weekly stimulation of CTLs for which perforin, granzyme B, granulysin and FasL RT-PCR were carried out.

Figures 5.9 a) to d) represent typical agarose gels obtained following RT-PCRs for perforin, granzyme B, granulysin and FasL. Figure 5.9 shows that CTL 20, 21, 22, 23 and 24 expressed mRNA transcripts for perforin, granzyme B, granulysin and FasL in phase 2 of growth, as RT-PCR products of the appropriate size were detected for each reaction. CTLs 15, 16, 17, 18 and 19 also expressed mRNA transcripts for perforin, granzyme B, granulysin and FasL in phase 2 of growth. The RT-PCR results for CTLs 5, 7 and 9 are given in table 5.6.

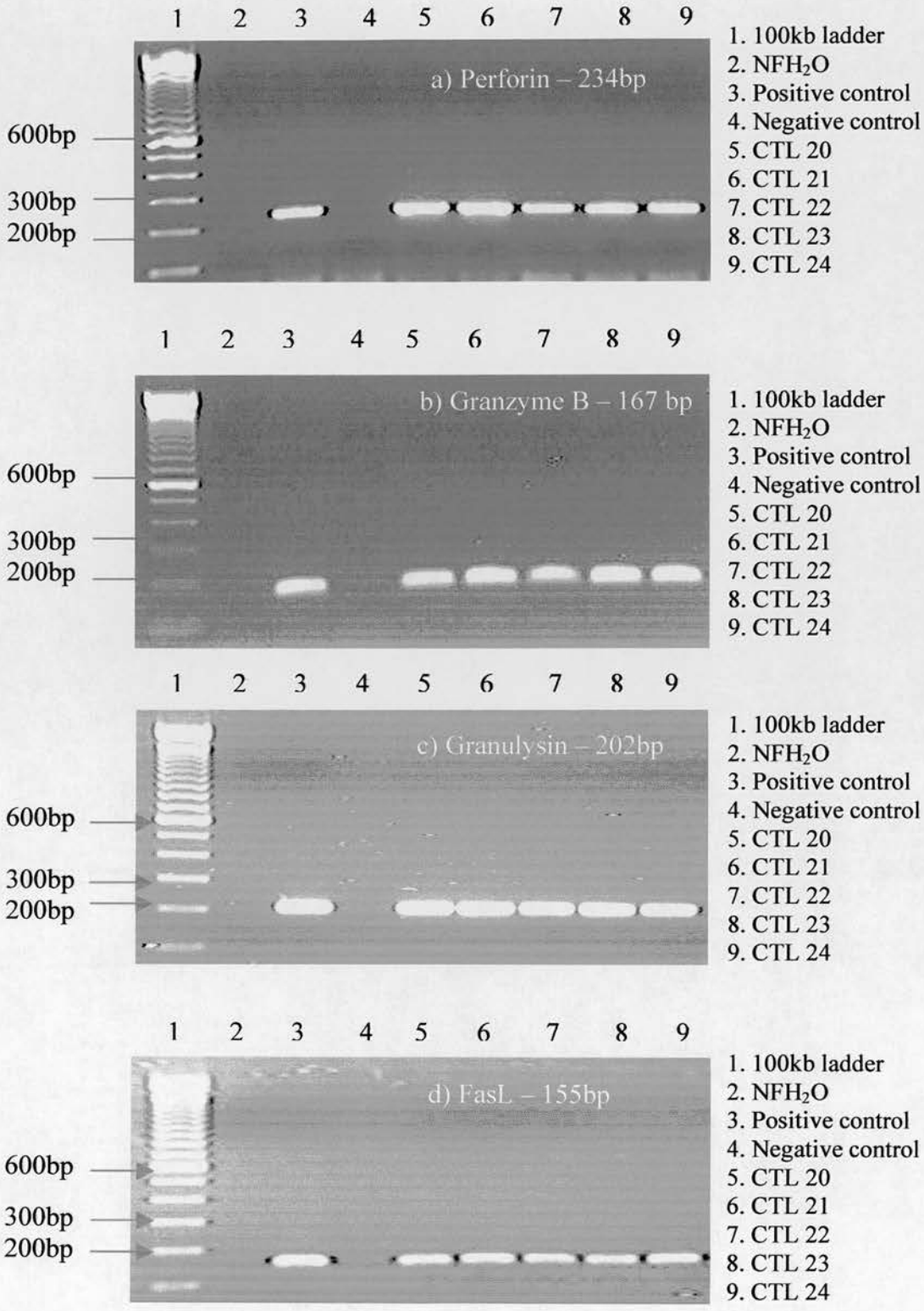


Figure 5.9: Agarose gels of a) perforin, b) granzyme B, c) granulysin and d) FasL RT-PCR on CTL 20, 21, 22, 23 and 24.

The results of all RT-PCRs carried out on CTLs 5, 7 and 9 (listed in table 5.5) are summarised in table 5.6 a) to d).

a) Perforin	S1	S2	S3	S4	S5	S6	S7
CTL 5	+	+	+	+	+	+	x
CTL 7	+	+	+	+	+	+	+
CTL 9	+	+	+	+	+	+	x

b) Granzyme B	S1	S2	S3	S4	S5	S6	S7
CTL 5	+	+	+	+	+	+	x
CTL 7	+	+	+	+	+	+	+
CTL 9	+	+	+	+	+	+	x

c) Granulysin	S1	S2	S3	S4	S5	S6	S7
CTL 5	+	+	+	+	+	+	x
CTL 7	+	+	+	+	+	+	+
CTL 9	+	+	+	+	+	+	x

d) FasL	S1	S2	S3	S4	S5	S6	S7
CTL 5	+	+	+	+	+	+	x
CTL 7	-	+	+	+	+	+	+
CTL 9	-	-	-	-	-	-	x

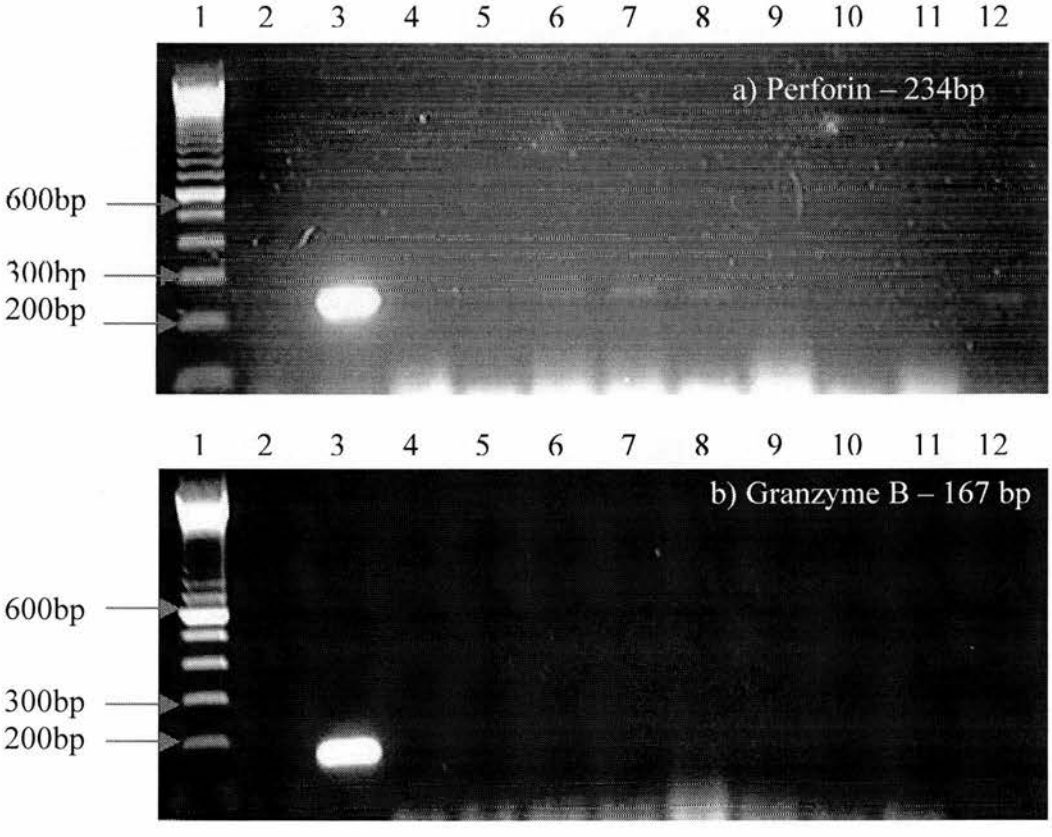
Table 5.6: Summary of RT-PCRs carried out on CTLs (listed in table 5.5). a) perforin, b) granzyme B, c) granulysin L and d) FasL.+: positive result, -: negative result, x: no sample available.

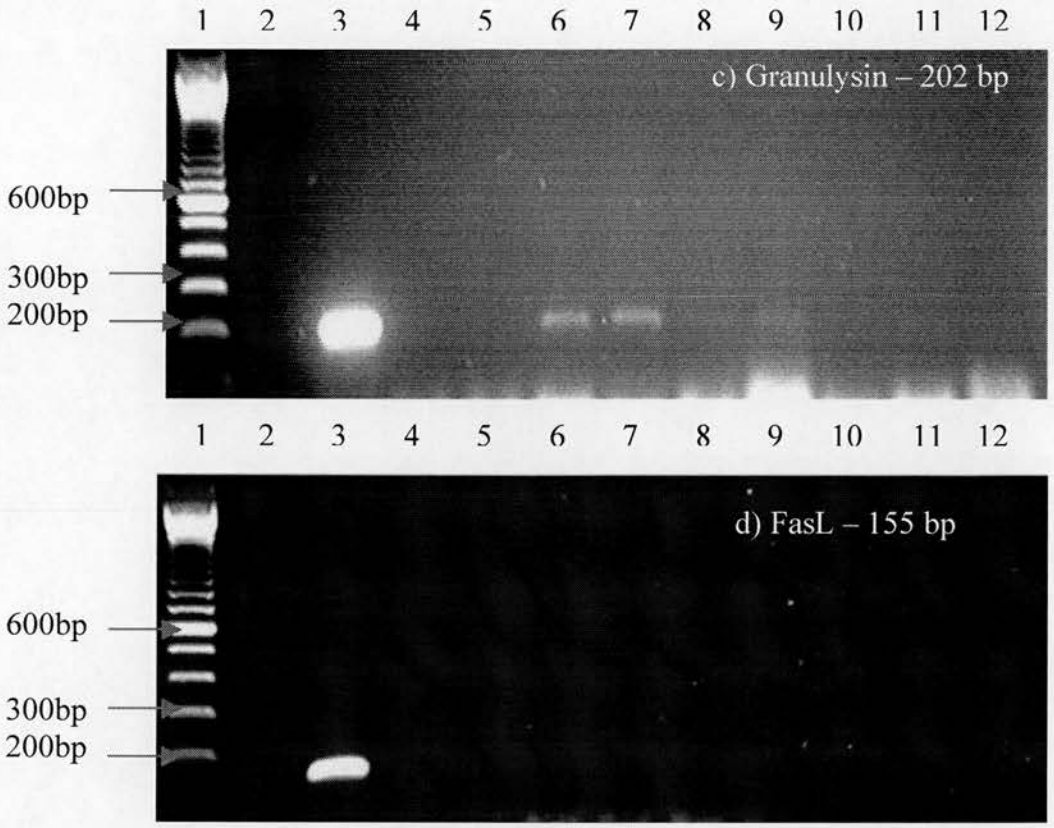
5.3.4 Perforin, granzyme B, granulysin and FasL RT-PCR on LCLs

As LCLs were used to stimulate the CTLs weekly during the course of *in vitro* culture and their prescence in the CTLs could not be entirely excluded, RT-PCRs were carried out on LCLs.

Figure 5.10 represents the agarose gels obtained following RT-PCRs for perforin, granzyme B, granulysin and FasL on LCLs. Some LCLs were used to stimulate multiple CTLs generated from the same PBMC donor. The numbers given to the LCLs correspond to the CTLs generated using these LCLs.

Agarose gel layout for perforin, granzyme B, granulysin and FasL RT-PCR			
1. 100bp ladder	4. Negative control	7. LCL 17	10. LCL 22
2. NFH_2O	5. K562 cells	8. LCL 5 & 18	11. LCL 23
3. Positive control	6. LCL 16	9. LCL 7 & 19	12. LCL 9 & 20





Figures 5.10: Agarose gels of a) perforin, b) granzyme B, c) granulysin and d) FasL RT-PCR on LCLs.

The results of all of the RT-PCR carried out on the LCLs are summarised in table 5.7, where "+" indicates a positive reaction and "-" indicates a negative reaction.

	Perforin	Granzyme B	Granulysin	Fas L
LCL 15	-	-	-	-
LCL 16	+	-	+	-
LCL 17	+	-	+	-
LCL 5 & 18	+	-	-	-
LCL 7 & 19	-	-	+	-
LCL 9 & 20	+	-	+	-
LCL 21	-	-	-	-
LCL 22	-	-	-	-
LCL 23	-	-	+	-
LCL 24	-	-	-	-

Table 5.7: Summary of RT-PCR results for LCLs.

5.3.5 Results summary

Perforin, granzyme B and granulysin mRNA transcripts were detectable throughout phases 1 and 2 of growth in CTLs 5, 7 and 9. However, FasL mRNA was not consistently detected throughout phases 1 and 2 of growth in CTLs 5 and 7 and it was not detected in phases 1 or 2 of growth in CTLs 9. mRNA transcripts for perforin, granzyme B, granulysin and FasL were detected in CTLs 15 to 24.

Perforin mRNA transcripts were detected in four of the seven LCLs tested and granulysin mRNA transcripts were detected in five of the seven LCLs tested (16, 17, 7 & 19, 9 & 20, 23). No mRNA transcripts for granzyme B and FasL were detected. This means that the mRNA transcripts detected in the CTLs may be of LCL origin and not of CTL origin. To overcome this, the expression of the cytolytic proteins was assessed next.

5.4 Expression of cytolytic proteins in the CTLs

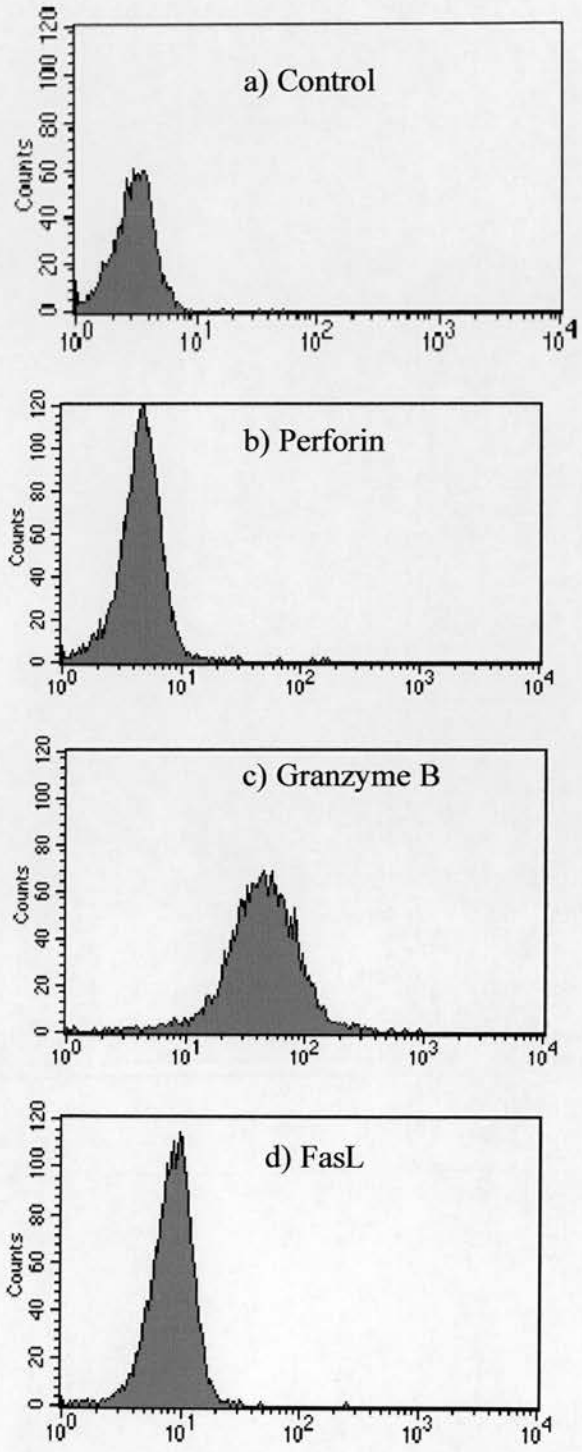
Based on the results obtained above, where mRNA transcripts were detected in LCLs, further experiments were carried out to detect the cytolytic molecules at the protein level in the CTLs. Detection of cytolytic proteins was carried out in phase 2 of growth.

Perforin, granzyme B and FasL were all detected by intracellular FACS staining as described in chapter 2 section 2.4. Granulysin was detected by western blotting as described in chapter 2 section 2.5.

5.4.1 Expression of perforin, granzyme B and FasL in CTLs

5.4.1.1 Expression of perforin, granzyme B and FasL in CTLs

The expression of perforin, granzyme B and FasL in the CTLs was investigated by determining the mean fluorescence intensity (MFI) of each cytolytic molecule. The MFI is determined by taking the geometric mean of the perforin, granzyme B and FasL expressing cells and subtracting from that the MFI of unstained control cells. The geometric mean is the antilog of the mean of the logarithm of the values and it is less likely to be affected by outliers than the mean (Swinscow and Campbell, 2002). The MFI is expressed as an arbitrary unit (AU). Representative histograms used to determine the MFI of each cytolytic molecule in one CTL are shown in figure 5.11. This was done for all CTLs and the results are given in table A5.6 in appendix A5. The data in table A5.6 is graphically represented in figure 5.12.



a) Control – MFI: 2.98 AU

b) Perforin test MFI =

4.51 (Perforin MFI)
- 2.98 (Control MFI)

1.53 AU

c) Granzyme B test MFI =

42.13 (Granzyme B MFI)
- 2.98 (Control MFI)

39.95 AU

d) FasL test MFI =

8.24 (FasL MFI)
- 2.98 (Control MFI)

5.26 AU

Figure 5.11: Histograms used to determine the MFI of cytolytic molecules in one CTL (CTL 17). Histograms a) unstained control b) perforin MFI, c) granzyme B MFI and d) FasL MFI.

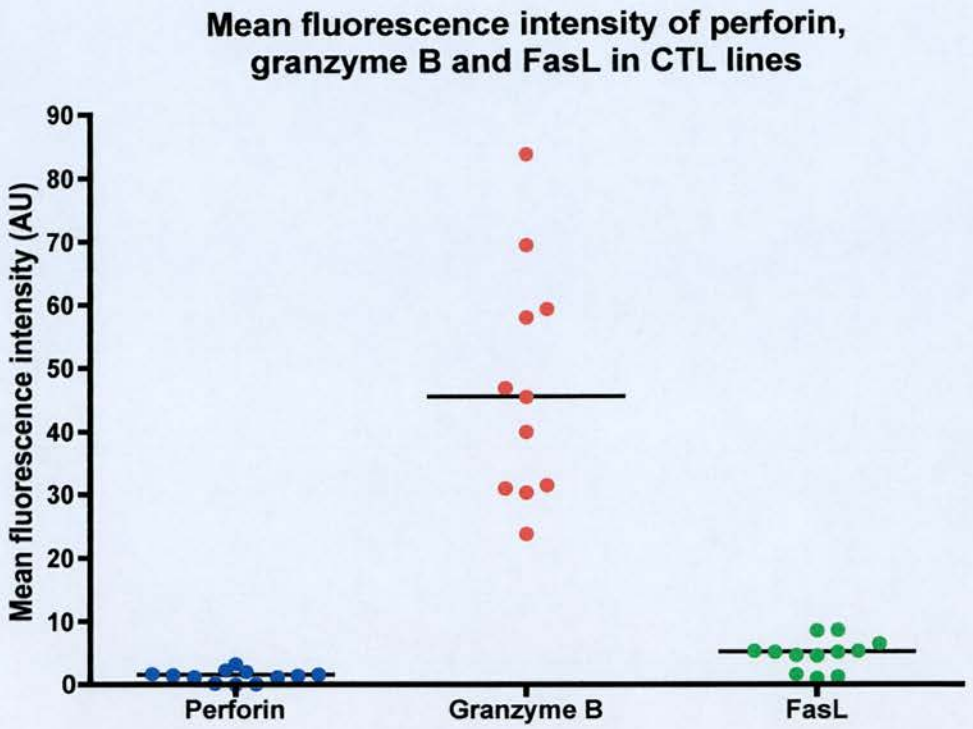


Figure 5.12: MFI of perforin, granzyme B and FasL in CTLs. AU: arbitrary units. n=12 (except for granzyme B n=11). The bold horizontal bar represents the median.

Result analysis

The results in table A5.6 and figure 5.12 show that there was considerably more granzyme B than FasL and perforin in the CTLs. There was also marginally more FasL than perforin in the CTLs.

5.4.1.2 Expression of perforin, granzyme B and FasL in the CD4+ T-cells and CD8+ T-cells of the CTLs

The expression of each cytolytic protein within the CD4+ T-cells and the CD8+ T-cells within the CTLs was next investigated. In order to do this "gates" were set around the CD4+ T-cell population (named R3 in figure 5.13) and around the CD8+ T-cell population (named R2 in figure 5.13), as shown in figure 5.13. The proportion of CD4+ T-cells and CD8+ T-cells in each of the CTLs was determined using this method and the results are in appendix A5 (table 5.10).

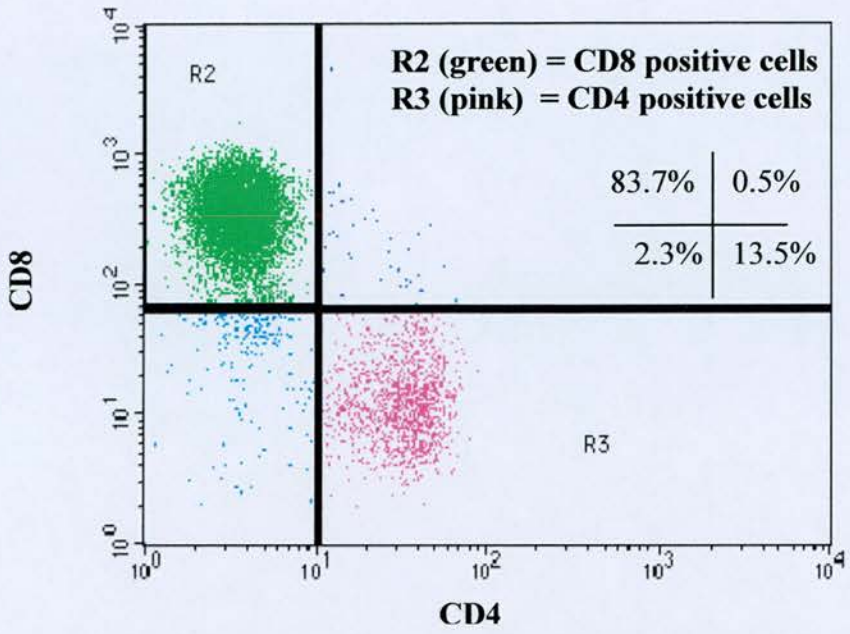
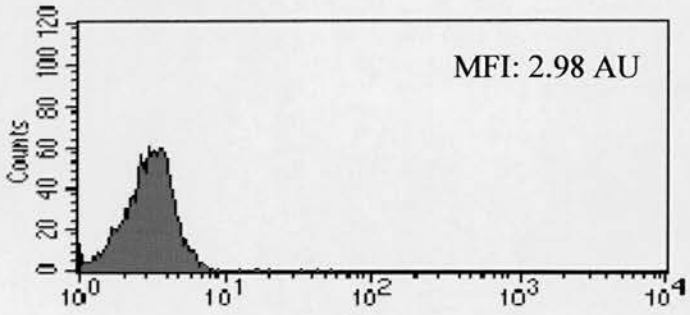


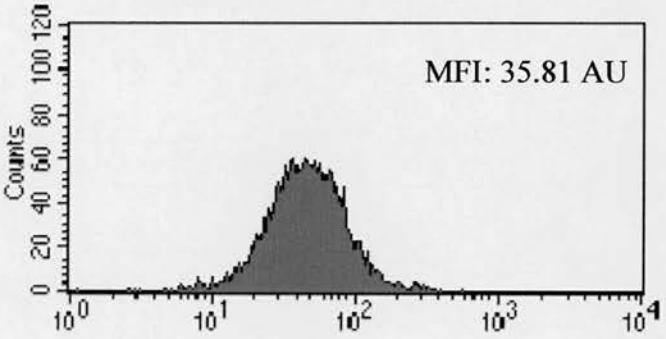
Figure 5.13: CD4+ T-cell and CD8+ T-cell gating in one CTL. R2 = upper left part of the quadrant (green cells) = CD8+ T-cells and R3 = lower right part of the quadrant (pink cells) = CD4+ T-cells. The values given in the quadrant are averages of three separate tests. This gating process was repeated for the data analysis of all CTLs. Once these gates were set, the expression of cytolytic proteins within the CD4+ and CD8+ T-cell populations was determined.

Figure 5.14 shows representative histograms of a) unstained control b) the granzyme B MFI in CD8+ T-cells c) the granzyme B MFI in CD4+ T-cells for one CTL. This figure also shows how the MFI of granzyme B in CD8+ T-cells and CD4+ T-cells was determined. This was done for all other CTLs and the MFI (for all cytolytic molecules) for the all of the CTLs are given in table A5.7 in appendix A5.

a) Control



b) Granzyme B in CD8+ T-cells

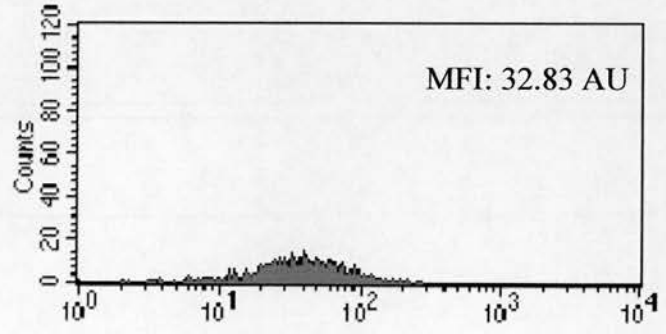


Test MFI =

44.28 (Granzyme B MFI) - 2.98 (Control MFI)

41.3

c) Granzyme B in CD4+ T-cells



Test MFI =

35.81 (Granzyme B MFI) - 2.98 (Control MFI)

32.83

Figure 5.14: Histograms used to determine the geometric mean of granzyme B in CD4+ T-cells and CD8+ T-cells in one CTL. a) control, b) granzyme B in CD8+ T-cells and c) granzyme B in CD4+ T-cells.

Figures 5.15 a) and b) represent the data in table A5.7.

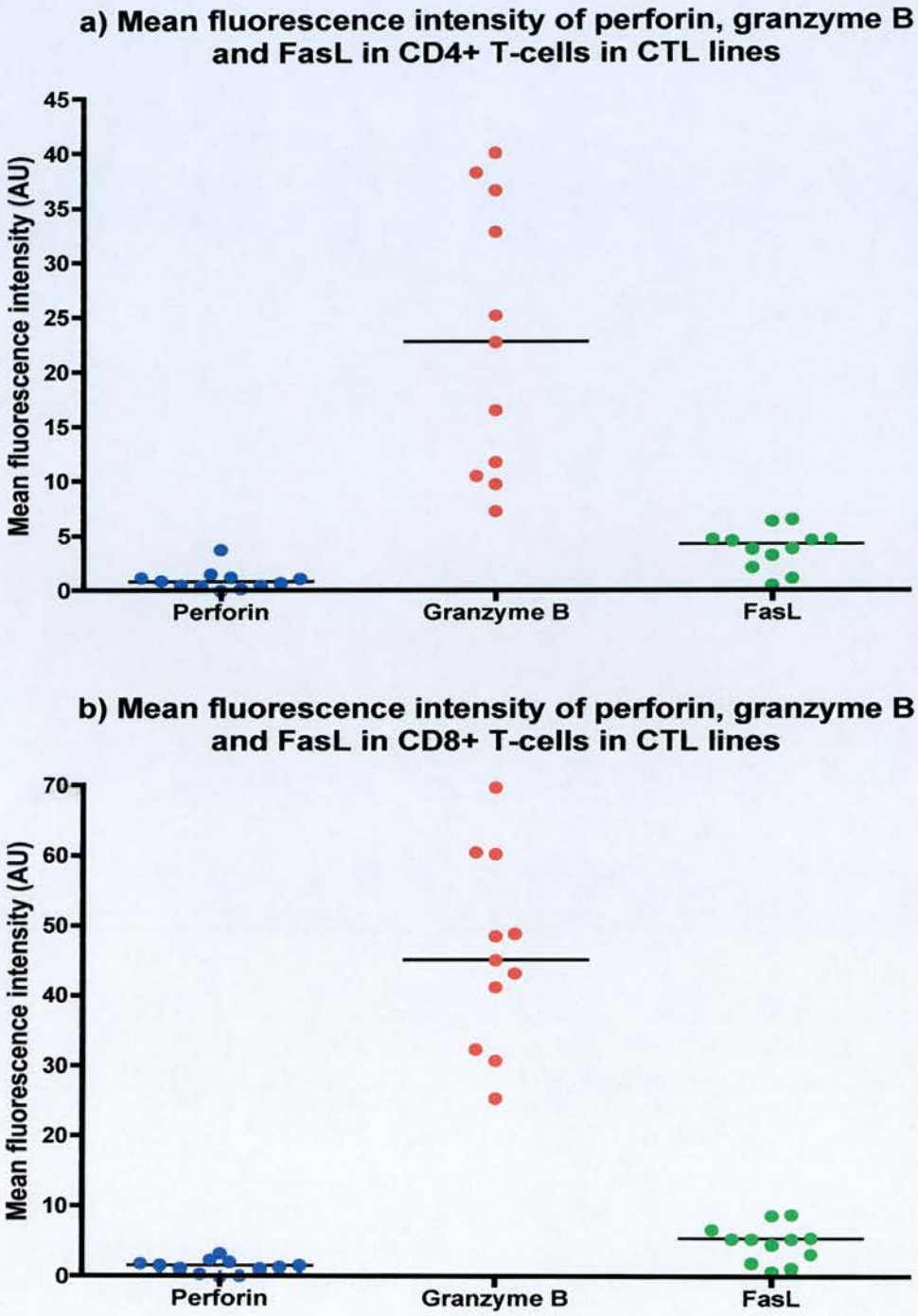


Figure 5.15: MFI of perforin, granzyme B and FasL in a) CD4+ T-cells and b) CD8+ T-cells. AU: arbitrary unit. n=12 (except for granzyme B n=11). The bold horizontal bar represents the median.

Result analysis

The results in table A5.7 and figure 5.15 parallel those found for the whole CTLs. They show that granzyme is most abundant in both CD4+ T-cells and CD8+ T-cells, followed by FasL and perforin.

The MFI of perforin, granzyme B and FasL were compared between CD4+ T-cells and CD8+ T-cells using the Mann-Whitney test. The results are given in table 5.8.

	Perforin MFI	**Granzyme B MFI**	**FasL MFI**
CD4+ T-cells vs CD8+ T-cells	$p = 0.17$	$p < 0.0016$*	$p = 0.26$

Table 5.8: Results of Mann-Whitney tests for perforin, granzyme B and FasL MFI between CD4+ T-cells and CD8+ T-cells in CTLs. * significant at $p < 0.05$.

These results show that there was no significant difference in the levels of perforin and FasL in CD4+ T-cells and CD8+ T-cells in the CTLs. However, there was significantly more granzyme B in CD8+ T-cells than in CD4+ T-cells in the CTLs.

5.4.1.3 Proportion of cytotoxic T-cells expressing perforin, granzyme B and FasL in the CTLs

The proportion of cells in the CTLs expressing perforin, granzyme B and FasL was determined next. This was done by defining a marker, based on the unstained control, where cells to left of this marker are negative and cells to the right of this marker are positive, as shown in figure 5.16 a) to d) for one CTLs. This was repeated for each of the CTLs.

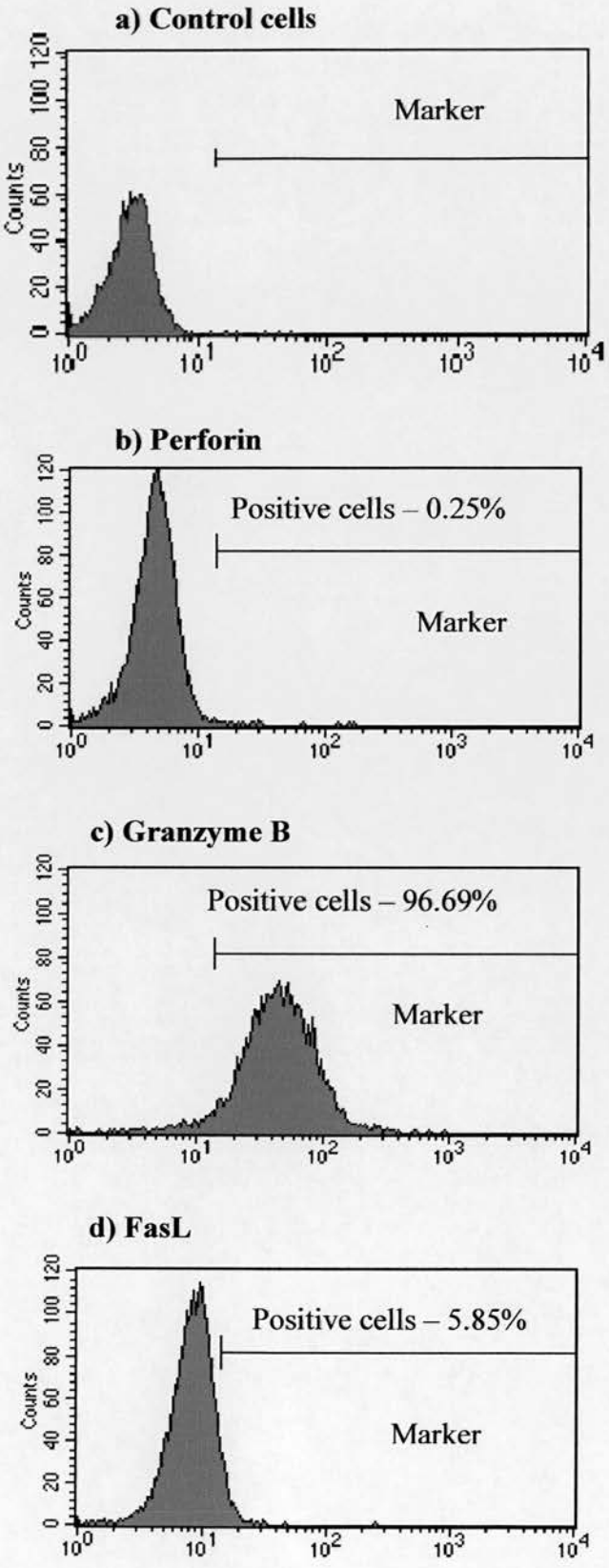


Figure 5.16: Proportion of cells expressing perforin, granzyme B and FasL in one CTL. a) control, b) perforin, c) granzyme and d) FasL.

This was done for all cytolytic molecules in all other CTLs and the proportion of cells expressing each cytolytic molecule is given in table A5.8 in appendix A5. Figure 5.17 represents data in table A5.8.

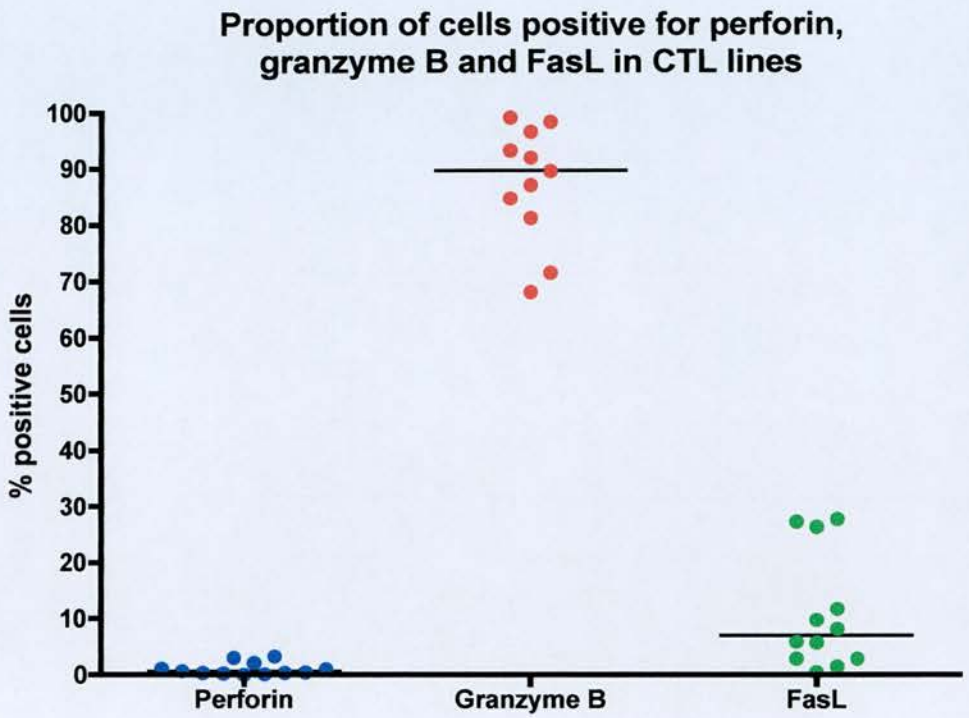


Figure 5.17: Proportion of cells expressing perforin, granzyme B and FasL in CTLs. n=12 (except for granzyme B n=11). The bold horizontal bar represents the median.

Result analysis

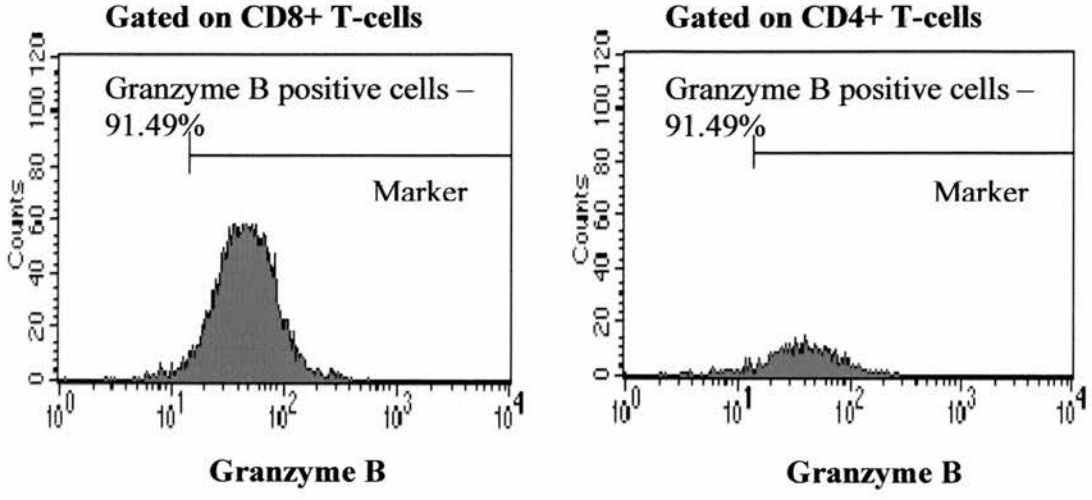
The results in table A5.8 and figure 5.17 show that a considerably greater proportion of the cells in CTLs expressed granzyme B than FasL or perforin. There was also a greater proportion of the cells in the CTLs expressing FasL and perforin.

5.4.1.4 Proportion of CD4+ T-cells and CD8+ T-cells expressing perforin, granzyme B and FasL in the CTLs

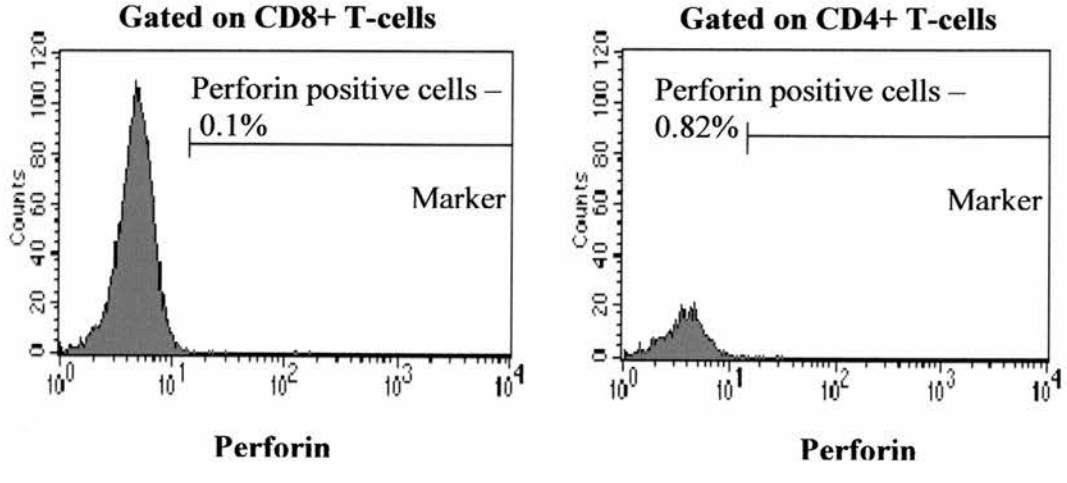
The proportion of CD4+ T-cells and CD8+ T-cells expressing cytolytic molecules was determined by using the same gating process as described in section 5.4.1.2 a) (see figure 5.13). The proportion of CD4+ T-cells and CD8+ T-cells expressing perforin, granzyme B and FasL in one CTL was determined by using the histograms

shown in figure 5.18 below (using the marker defined in figure 5.16). This was repeated for all CTLs and the results are given in table A5.9 in appendix A5. The data in table A5.9 is graphically represented in figure 5.19.

a) Proportion of granzyme B positive cells CD4+ T-cells and CD8+ T-cells



b) Proportion of perforin positive cells CD4+ T-cells and CD8+ T-cells



c) Proportion of FasL positive cells CD4+ T-cells and CD8+ T-cells

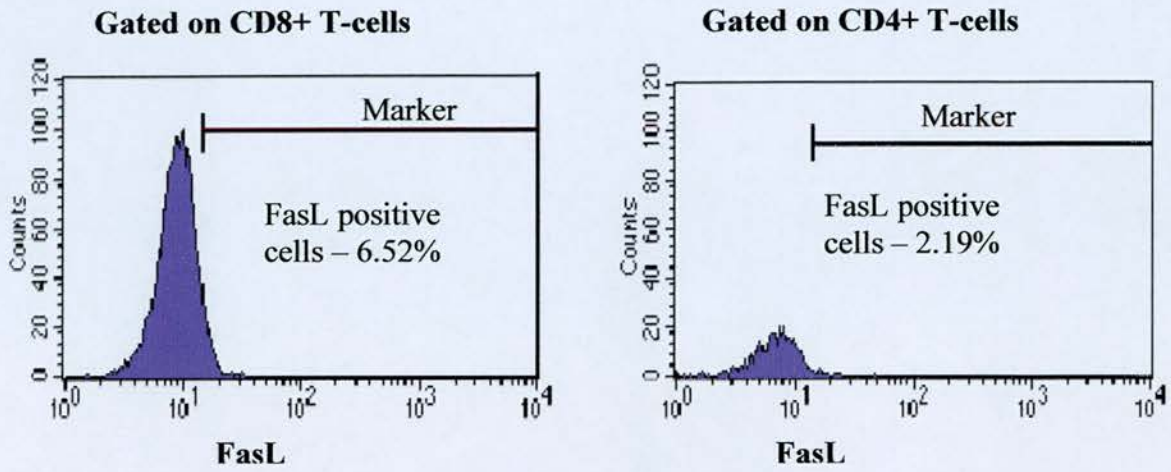
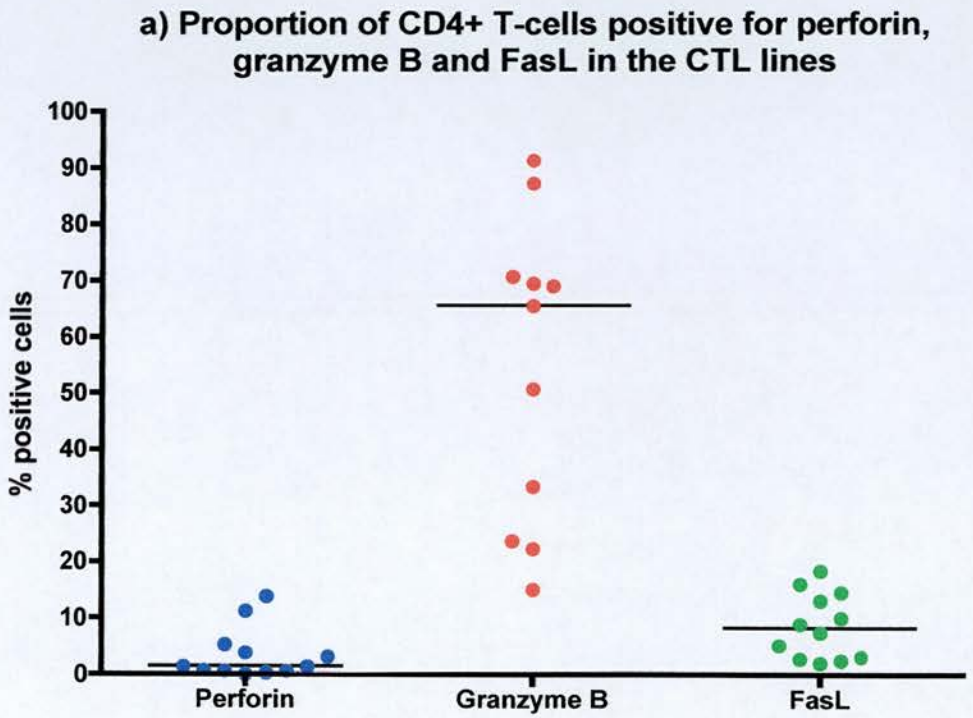


Figure 5.18: Proportion of CD4+ T-cells and CD8+ T-cells from CTL 3 expressing a) granzyme B, b) perforin and c) FasL.



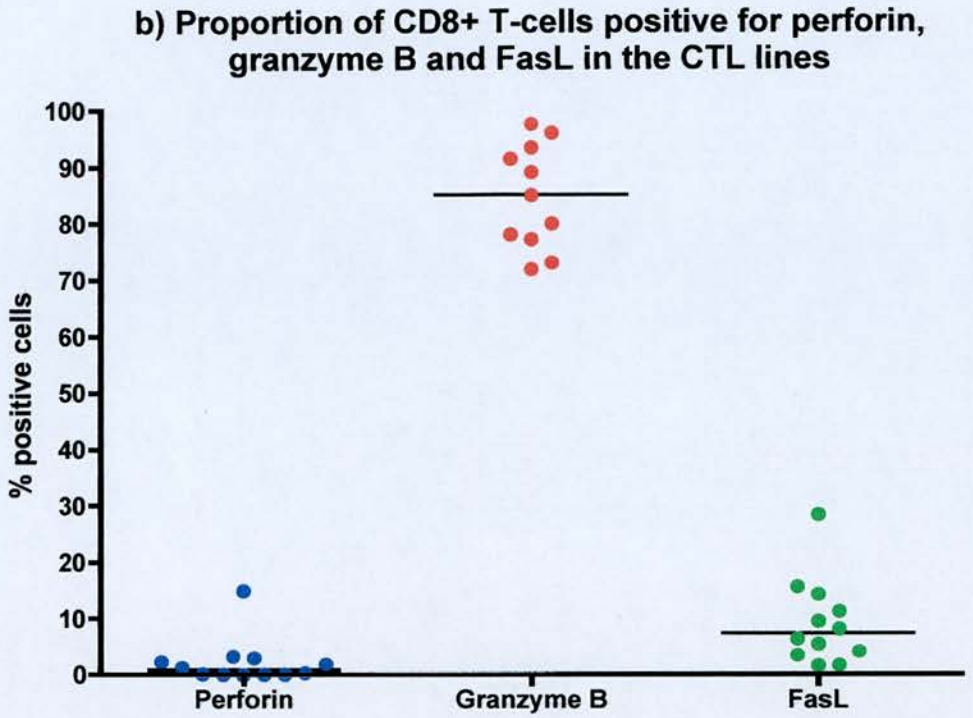


Figure 5.19: Proportion of a) CD4+ T-cells and b) CD8+ T-cells expressing perforin, granzyme B and FasL in all CTLs. n=12 (except for granzyme B n=11). The bold horizontal bar represents the median.

Result analysis

The results in table A5.9 and figure 5.19 parallel those found for the whole CTL. There was a considerably greater proportion of both CD4+ and CD8+ T-cells expressing granzyme B than FasL and perforin. In turn there was a greater proportion of CD4+ and CD8+ T-cells expressing FasL than perforin.

Mann-Whitney tests were carried out to compare whether there was a difference in the proportion of CD4+ T-cells and CD8+ T-cells expressing perforin, granzyme B and FasL in the CTLs. The results of the Mann-Whitney tests are given in table 5.9.

	Perforin	Granzyme B	FasL
CD4+ T-cells vs CD8+ T-cells	p = 0.19	p = 0.0023*	p = 0.93

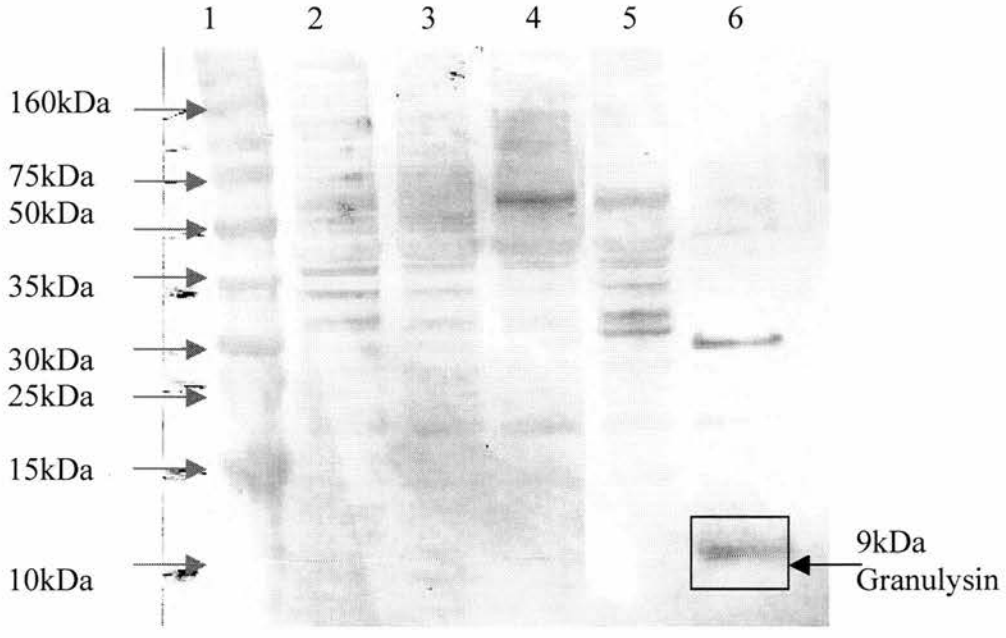
Table 5.9: Results of Mann-Whitney tests between the proportions of CD4+ T-cells and CD8+ T-cells expressing perforin, granzyme B and FasL. * significant at $p < 0.05$.

These results show that there was no significant difference in the proportion of CD4+ T-cells and CD8+ T-cells expressing perforin or FasL. However, they show that there was a significantly greater proportion of CD8+ T-cells expressing granzyme B than there were CD4+ T-cells expressing granzyme B in the CTLs.

5.4.2 Detection of granulysin in CTLs

5.4.2.1 Western blots for granulysin in LCLs

As LCLs 7, 9, 16, 17 and 23 tested positive by RT-PCR for granulysin mRNA transcripts, all the LCLs used to stimulate the CTLs were tested for the expression of granulysin protein. CD56-expressing cells were used as a positive control. This experiment was carried out as described in section 2.5. Figure 5.20 shows a representative western blot for granulysin carried out on four LCLs.



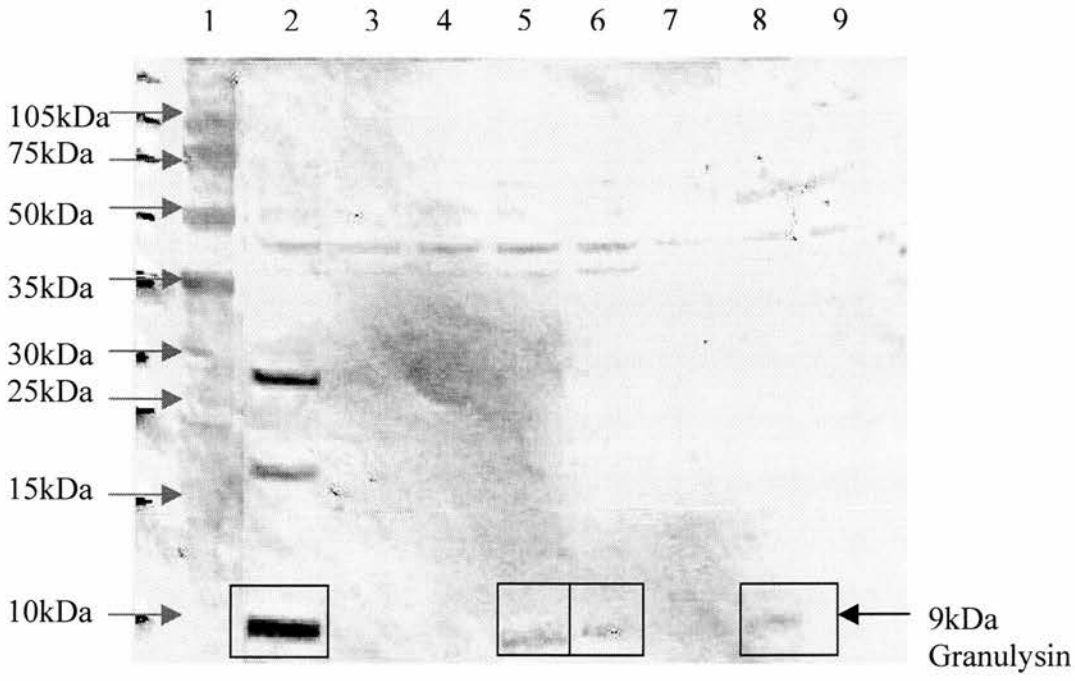
1. Rainbow ladder	4. LCL 17
2. LCL 15	5. LCL 7 & 19
3. LCL 16	6. Positive control

Figure 5.20: Western blot of LCLs 15,16, 17 and 7 & 19 for granulysin detection.

This figure shows that there is a clear and strong band at 9kDa for the positive control, which corresponds to the granulysin protein. The other band for the positive control at 27kDa is likely to be a complex of three granulysin proteins, as these can cluster (Krensky and Clayberger, 2005). There is no band at 9kDa for any of the LCLs indicating that no granulysin could be detected within these cells. No granulysin was detected by western blotting in LCLs 9 & 20, 21, 22, 23 and 24.

5.4.2.2 Western blots for granulysin in CTLs

As granulysin could not be detected in the LCLs, all the CTLs were tested. CD56-expressing cells were used as a positive control. Figure 5.21 is a representative western blot of seven of the CTLs tested.



1. Rainbow ladder	4. CTL 22	7. CTL 5
2. Positive control	5. CTL 23	8. CTL 7
3. CTL 21	6. CTL 24	9. CTL 9

Figure 5.21: Western blot of CTLs 5, 7, 9, 21, 22, 23 and 24 for granulysin detection.

This figure again shows that there was a clear and strong band at 9kDa for the positive control, which corresponds to granulysin, as well as bands at 18kDa and 27kDa corresponding to granulysin complexes. No granulysin could be detected in CTLs 5, 9, 21 and 22, however a 9kDa band was detected for CTL 7, 23 and 24. Granulysin was also detected in CTL 19 but it was not detected in CTLs 15, 16, 17 and 20.

5.4.3 Results summary for cytolytic protein detection in CTLs

There was considerably more granzyme B than FasL and in turn there was more FasL than perforin in the CTLs. This was also the case in the CD4+ T-cells and the CD8+ T-cells within the CTLs. There was significantly more granzyme B in CD8+ T-cells than in CD4+ T-cells but there was no significant difference in the amount of perforin and FasL between the two cell types. There was also a considerably greater proportion of cells in the CTLs, which were expressing granzyme B than FasL and in turn there were more cells expressing FasL than perforin. This was also seen in CD4+ T-cells and CD8+ T-cells within the CTLs. There was a significantly greater proportion of CD8+ T-cells than CD4+ T-cells expressing granzyme B but there was no significant difference in the proportion of CD4+ T-cells than CD8+ T-cells expressing perforin or FasL. Granulysin was detected in four CTLs (33% of CTLs). It was not detected in LCLs.

5.5 Discussion

5.5.1 Cytotoxic pathways

The standard 4-hour cytotoxicity assays showed that the CTLs were all specific for their autologous LCLs, with comparatively low killing of the HLA-mismatched LCLs and K562 cells. Haque *et al* (2002) set cut-off values to determine whether a CTL was sufficiently specific for infusion in PTLD patients. These were >30% of autologous LCL lysis, <10% HLA-mismatched LCL and K562 cells. The results of the standard cytotoxicity assays given in table 5.1 and figure 5.1 show that 10 (77%) of the 13 CTLs tested exhibited >30% autologous LCL lysis and that three did not (CTLs 16, 21 and 22) at E:T 20:1. Their autologous LCL lysis though was >20%. Nine (70%) of the CTLs exhibited <10% HLA-mismatched LCL lysis. All CTLs, with the exception of one, exhibited <10% K562 cell lysis. Whilst the cytotoxicity results for some of the CTLs tested might not fulfil the strict criteria set up by Haque *et al* (2002), they do demonstrate that all of the CTLs exhibit greatest lysis towards their autologous LCL, which would be indicative of target specificity. Hence, the cytotoxic pathway(s) employed by these CTLs were investigated using the modified 4-hour chromium release cytotoxicity assay.

The modified 4-hour cytotoxicity assays showed that cytotoxicity was significantly inhibited by EGTA ($p=0.0007$ at E:T 20:1) and CMA ($p<0.0001$ at E:T 20:1) at all three E:T ratios. (Both inhibitors are known to be non toxic to cells (Yasukawa *et al*, 2000)). On the other hand BFA did not significantly reduce cytotoxicity ($p=0.2855$ at E:T 20:1). These findings indicate that the cytotoxic pathway(s) of the CTLs is calcium-dependent as EGTA, a calcium-chelating agent, significantly inhibited cytotoxicity in all CTLs tested. CMA also significantly inhibited cytotoxicity in all CTLs tested. CMA is a specific inhibitor of perforin that acts by preventing the acidification of vacuoles containing perforin, which leads to its degradation (Kataoka *et al*, 1996; Kataoka *et al*, 1994). This suggests that perforin and also granzyme B,

which is found in conjunction with perforin, are key cytotoxic proteins in the CTLs. BFA did not significantly reduce cytotoxicity, indicating that Golgi-mediated transport of cytolytic proteins did not significantly contribute to cytotoxicity of the CTLs. Whilst these cytotoxicity assays provide strong indications of which pathways are employed by the CTLs experiments were carried out to determine whether perforin, granzyme B and FasL were present in the CTLs. In addition to these, granulysin was also studied, as it has been suggested that it may add to the activity of perforin (not in a synergistic fashion) (Kaspar *et al*, 2001).

5.5.2 Cytolytic proteins

RT-PCR results showed that all but one of the CTLs expressed mRNA transcripts for all four cytolytic proteins (CTL 9 did not express FasL mRNA at any of the stimulations tested). FACS and western blotting were used to complement the RT-PCR results, not only in order to detect the proteins themselves but also because mRNA transcripts of the cytolytic molecules were detected in LCLs.

The proposed models of differentiation of EBV-specific CD4+ and CD8+ T-cells indicate that, along with the downregulation in CD27 and CD28 expression, both cell types acquired perforin expression (Appay *et al*, 2002a; Appay *et al*, 2002b). However, FACS analysis of the CTLs showed that granzyme B was the most abundant cytolytic molecule, with FasL and perforin being considerably less abundant (Figure 5.12). So, whilst both cell types showed downregulation in CD27 and CD28 expression, as shown in chapter 3, there was no concomitant increase in perforin expression. In healthy blood donors the average proportion of CD4+ T-cell expressing perforin was 2.2±0.6% (Appay *et al*, 2002b). The average proportion of CD4+ T-cell expressing perforin in the CTLs was 3.6% and the average proportion of CD8+ T-cells was lower at 3.4%. This suggests that small amounts of perforin are sufficient for cytotoxicity to take place.

The modified 4-hour cytotoxicity assays showed that effector functions of the CTLs were calcium-mediated and perforin-mediated as EGTA and CMA both inhibited cytotoxicity. This shows that perforin is pivotal in CTL cytotoxicity, as has previously been shown (Trapani and Sutton, 2003). This suggests that granzyme B is responsible for the cytotoxicity exhibited by the CTLs and that a small amount of perforin is both necessary and sufficient for target cell killing to occur. On the other hand it appears that FasL plays only a minor role in CTL cytotoxicity. BFA did not inhibit cytotoxicity indicating that transcriptional upregulation and subsequent Golgi-mediated transport of FasL did not significantly contribute to the cytotoxicity achieved by the CTLs. However, FasL expression, at very low levels, can be induced by rapid transport and degranulation of pre-formed stores found in cytotoxic granules along with perforin and granzyme B (Bossi and Griffiths, 1999). It follows that pre-formed FasL is released/expressed in a calcium-dependent dependent fashion, but, due to the very small amounts found in these stores, this mechanism is unlikely to contribute greatly to cytotoxicity comparatively to granzyme B.

In summary, granzyme B is the cytolytic molecule found most abundantly in the CTLs and clearly plays a key role in their killing of autologous LCLs. Perforin also has a key role in LCL lysis, as demonstrated by the significant inhibition effected by CMA. The function of perforin may be as a permissive factor – its presence in small amounts being required for granzyme B activity. The importance of FasL is less clear, as inhibition of its upregulation had no significant effect on cytotoxicity suggesting that it plays at most a minor part in the process. The presence of CD4+ T-cells expressing cytolytic proteins, in particular granzyme B, indicate that these cells have the potential to contribute to the overall cytotoxicity of the CTLs, the bulk of which will be associated with the CD8+ T-cells. Supporting this finding is that PTLD patients treated with allogenic CTLs containing a higher mean percentage of CD4+ T-cells responded better than those treated with CTLs that contained fewer CTLs (Wilkie *et al*, 2004). This is substantiated further by Haque *et al* (manuscript in preparation).

Chapter 6

Discussion

6.1 Introduction

6.2 Growth, phenotype and cytotoxicity of CTLs

6.3 CTL proteins and cytolytic pathways

6.4 CD4+ T-cells and CD8+ T-cells in the CTLs

6.5 Conclusions

6.6 Future developments

6.1 Introduction

This project set out to investigate the growth and phenotypic development of EBV-specific CTLs, as well as their TCR repertoire diversity. Additionally, the presence of cytolytic molecules and the pathways by which the CTLs effect their cytotoxic activity were also investigated. This was carried out in order to appreciate and understand how EBV-specific CTLs, which are in use in the clinical setting, evolve in culture in response to weekly autologous LCL stimulations. Basic characterisation of CTLs may enhance understanding of potentially essential features of CTLs, which may need to be taken into consideration for the generation of clinically effective CTLs.

Adoptive T-cell immunotherapy for the successful treatment of PTLD using EBV-specific CTLs was pioneered by Rooney *et al* (1995). Following the same principle, other investigators used autologous and allogeneic CTL therapy to treat PTLD, although this was achieved on a small scale in terms of CTL numbers. Wilkie *et al* (2004) established a CTL bank, using the same method as Rooney *et al* (1995), consisting of over 100 CTLs covering the majority of HLA-types found in the local population. The establishment of this bank aimed to overcome the time required to generate autologous CTLs in order to provide prompt PTLD treatment. CTL generation time however, remains a critical issue, as it may take in excess of three months to generate antigen specific CTLs.

6.2 Growth, phenotype, genotype and cytotoxicity of CTLs

6.2.1 Phenotype and growth of CTLs

Phenotypic analysis of the CTLs during the course of their development showed that the basic phenotype of the CTLs, based around CD4 and CD8 expression, was determined in the first three to four weeks of culture. Concurrently, the CTLs also progressed towards an antigen-experienced phenotype characterised by an increase in CD45RO and CD69 expression and a decrease in CD27, CD28, CD45RA and CD62L. These phenotypic changes remained apparent later on in culture. In these further differentiated CTLs (including both CD4+ T-cells and CD8+ T-cells) granzyme B and perforin were detected in CTLs, with granzyme B detected at high level in CD8+ T-cells and lower level in CD4+ T-cells and perforin detected at low level in both cell types. FasL expression was low in both cell types. This shows that the culture system used, efficiently induced phenotypic changes typical of an antigen-experienced/effector phenotype in the early part of culture. This system also promoted the maintenance of these changes in the CTLs but that this was not accompanied by a pronounced increase in perforin levels, unlike CD4+ and CD8+ T-cells specific for EBV *in vivo* (Appay, 2004; Appay and Rowland-Jones, 2004).

The phenotypic development of the CTLs was clearly a dynamic process. Such dynamics were also seen in the proportional growth pattern and viability of the CTLs. There was an increase in proportional growth between S2 and S4, following a comparative decrease week on week, with CTLs no longer reaching the $1x10^6$ cells/ml benchmark after 10 weeks in culture. This pattern of proportional growth conforms to that previously reported by Wilkie *et al* (2004). The viability of the CTLs decreased from S2 onwards and decreased more rapidly after S4. Collectively, these results indicate that there is a limit to the potential growth that the CTLs could achieve. This may be related to the expression of $p16^{INK4}$, a protein essential in the

control of cell proliferation, the expression of which is associated with cells exiting the cell proliferation cycle (Migliaccio *et al*, 2005). Another potential factor in the limited growth of the CTLs *in vitro* may be related replicative senescence, which occurs when telomere shortening becomes critical that it is no longer possible for the cell to divide appropriately, at which point cell cycle arrest occurs. Transduction of CTLs with telomerase reverse transcriptase may be a way of overcoming replicative senescence, although this has been associated with genetic aberrations in particular cell lines and where abnormalities were absent. However, immunological senescence occurred following 12 months of culture (Schreurs *et al*, 2005).

Whilst the CTLs could not be cultured indefinitely, the culture system used for the generation of the CTLs induced their appropriate phenotypic and functional development, as discussed above and in section 6.2.3.

6.2.2 TCR repertoire of CTLs

Antigenic stimulation of the TCR underlies the phenotypic changes occurring in the CTLs discussed above. The TCR repertoire of the CTLs investigated remained diverse both in terms of Vβ family usage (i.e. the number of families in use) and in terms of Vβ family clonality, as there was no discernible pattern either in reduction of Vβ family usage or in emergence of clonal Vβ families during the course of culture. The development and maintenance of the TCR repertoire appears to differ from what is seen in IM. In IM, the primary response to EBV, there is an expansion of clonal and oligoclonal populations of CD8+ T-cells, which may be essential in the control of the infection (Callan *et al*, 1996). This is unlike the TCR development of the CTLs cultured here, which did not appear to clonally expand in the initial phase of culture, although the addition of IL-2 to maintain T-cell proliferation may have obscured the emergence of clonal or oligoclonal populations. Although, individuals undergoing silent seroconversion maintained TCR repertoire diversity without the detection of clonal/oligoclonal expansions (Silins *et al*, 2001). This suggests that

primary EBV response may not always be associated with specific TCR repertoire expansions.

T-cell repertoire diversity maintenance may be due to LCLs exhibiting prominent peptide-MHC features, the absence of which has been shown to limit repertoire diversity in virus-specific CD8+ T-cells (Turner *et al*, 2005). In addition, the TCR can recognise different peptides presented by the same MHC molecule (Reiser *et al*, 2003), which may result in the CTLs recognising a greater number of peptides in an allogeneic context. This would be a beneficial feature of allogeneic CTLs used for the treatment of PTLD, increasing the likelihood of an antigenic peptide being recognised by the CTLs. This shows that autologous LCL stimulations maintained TCR diversity, which strongly supports the establishment and use of a CTL bank covering many HLA types (Wilkie *et al*, 2004), as one CTL may therefore be used for multiple patients in an allogeneic setting.

6.2.3 Cytotoxicity of the CTLs

The phenotypic changes observed in the first four weeks of culture, as well as in subsequent weeks, were associated with differentiation of the CTLs towards an antigen-experienced/mature phenotype with the acquisition of cytolytic effectors. The results of the standard 4-hour cytotoxicity chromium release assays confirmed that the CTLs were cytotoxic and specific following at least five weeks of culture. This occurred without any apparent restrictions in the TCR repertoire of the CTLs.

Having established that the CTLs were cytotoxic and specific towards autologous LCLs, preliminary experiments were carried out to determine the antigen specificity of the CTLs. This was done using dendritic cells infected with recombinant vaccinia vectors encoding EBV antigens, as antigen presenting cells. These antigen presenting cells were used as target cells in a 4-hour cytotoxicity assay. The percentage of cell lysis was used as a measure of antigen specificity. This assay was carried out as described in section 2.2. Three CTLs were tested: CTL 7, CTL 19 and CTL 20.

CTLs 19 and 20 were tested by Karen McAulay. Figure 6.1 represents the results of a vaccinia 4-hour chromium release cytotoxicity assay carried out using CTL 7.

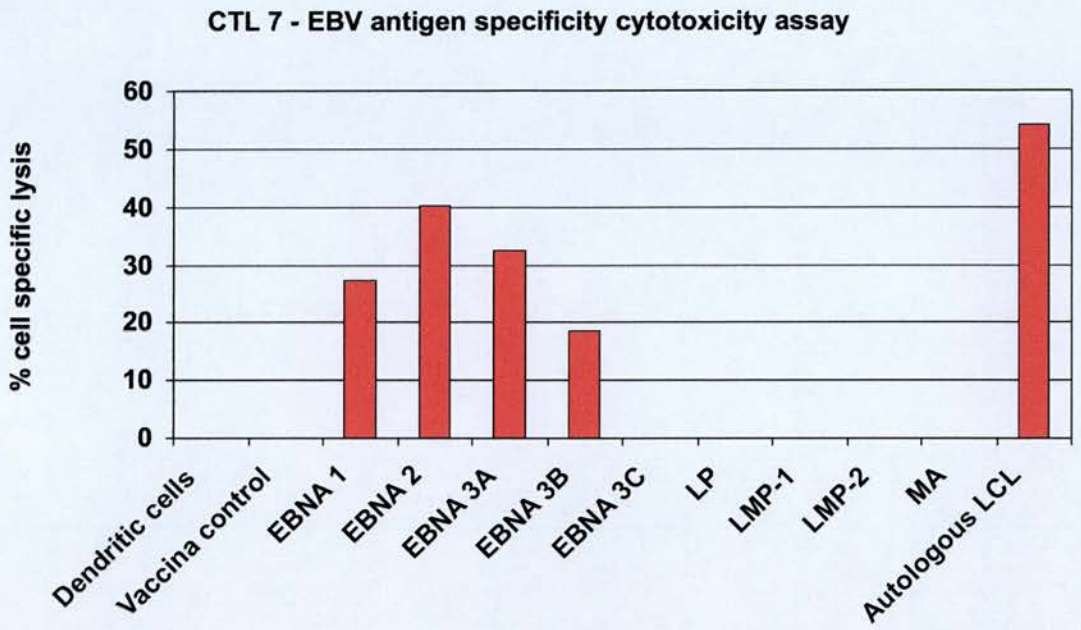


Figure 6.1: Results from 4-hour vaccinia chromium release cytotoxicity assay using CTL 7.

This figure shows that the CTL was cytotoxic and therefore specific towards EBNA-1, EBNA-2, EBNA-3A and 3B. The CTL was not cytotoxic towards the uninfected dendritic cells or the vaccinia control. The cytotoxicity results for the three CTLs tested are summarised in table 6.1.

	CTL 7	CTL 19	CTL 20
Dendritic cells	0%	2.2%	0.4%
Vaccinia control	0%	0%	0%
EBNA-1	27.3%	4.8%	0.1%
EBNA-2	40.3%	0%	0%
EBNA-3A	32.5%	0%	0%
EBNA-3B	18.6%	55.9%	0%
EBNA-3C	0%	0%	20.1%
EBNA-LP	0%	17.3%	11.5%
LMP-1	0%	19%	0.1%
LMP-2	0%	22.7%	0%
MA	0%	0%	0%
Autologous LCL	54.1%	25.2%	24%

Table 6.1: CTL antigen specificity.

These preliminary results show that the three CTLs tested were distinctly specific to particular antigens, whilst not being specific to others. All three CTLs were specific towards one or two of the three EBNA-3 antigens known to be immunodominant among the EBV latent proteins. The CTLs 19 and 20 were also specific towards EBNA-LP.

Characterising the antigen specificity of CTLs may enable more targeted treatment of PTLD by matching tumour antigen expression and CTL antigenic specificity.

6.3 CTL proteins and cytolytic pathways

The effector functions gained by the CTLs during the course of culture appeared to be centred on perforin, found to be essential and necessary to cytotoxicity, and granzyme B, the most abundant of the three cytolytic molecules investigated by intracellular FACS staining in both CD4+ and CD8+ T-cells. In healthy blood donors the average proportion of CD4+ T-cell expressing perforin was 2.2±0.6% (Appay *et al*, 2002b). The average proportion of CD4+ T-cell expressing perforin in the CTLs was 3.6% and the average proportion of CD8+ T-cells was lower at 3.4%. Furthermore, the relative paucity of perforin in comparison to granzyme B was surprising as chronic EBV antigenic stimulation *in vivo* (in EBV carriers) results in increased expression of perforin in both CD4+ T-cells and CD8+ T-cells analysed *ex vivo* (Appay *et al*, 2002a; Appay *et al*, 2002b). This suggests that prolonged *in vitro* culture with autologous LCL stimulation does not induce an increase in the proportion of cells expressing perforin (or perforin expression) but rather an increase in the proportion of cells expressing granzyme B (0.6% CD4+ T-cells and 10.4% CD8+ T-cells expressed granzyme B in PBMC (Grossman *et al*, 2004)). Low level perforin expression however, did not to translate into reduced target cell cytotoxicity as target cell lysis still occurred in chromium release cytotoxic assays, indicating that granzyme B was still able to effect its pro-apoptotic role in the target cells (as granzyme B is essential in CTL cytotoxicity (Heusel *et al*, 1994)). A number of mechanisms by which granzyme B reaches the target cells have been proposed, all of which crucially remain dependent on perforin and are not mutually exclusive. The initial mechanism put forward involved perforin creating pores in the cell membrane allowing granzyme B to diffuse passively through to the target cells and induce apoptosis (Trapani and Smyth, 2002). Subsequently, a pathway was proposed involving the internalisation of perforin pores (to which granzyme B was bound) from the target cell surface into an endosome where the pores would facilitate the release of granzyme B in the target cell (Browne *et al*, 1999). However, the same group subsequently proposed a model in which many granzyme B molecules were

delivered to the target cell bound to serglycin (a proteoglycan) with perforin either bound to the serglycin/granzyme B complex or in a monomeric form. In this model, the target cell became apoptotic in the absence of plasma membrane pores (suggesting that perforin was present at sub-permeabilising amounts), indicating that the serglycin/granzyme B complex was internalised by the target cell (Metkar *et al*, 2002). A recent publication proposed a further model in which granzyme B, as well as other cytolytic/cationic proteins (including granulysin), dissociated from serglycin at the surface of the target cell, and entered the target cell by pinocytosis or endocytosis mediated by binding to cell surface proteoglycan, subsequently leading to lysis of the endosome and apoptosis (Raja *et al*, 2005). Aside from the initial model described, these models suggested that perforin is not required in high concentrations (sub-permeablising) to facilitate the entry of many granzyme B (complexed or not to serglycin) into a target. This may be why the CTLs investigated in this project were cytotoxic even though perforin was only present at low level and granzyme B was considerably more abundant.

Granulysin, a saposin-like molecule found in cytolytic granules (Krensky and Clayberger, 2005), was detected in 33% of CTLs tested by western blotting. Research by Kaspar *et al* (2001) indicated that granulysin may act in a summative fashion with perforin, leading to increased membrane damage and ultimately to mitochondrial damage and apoptosis. This suggests that the granulysin found in the CTLs may be contributing to their cytotoxicity towards autologous LCLs. Raja *et al* (2002) hypothesised that granulysin may be bound to serglycin along with granzyme B and perforin in which case the role and function of granulysin may be perforin-dependent.

FasL on the other hand, did not appear to contribute greatly to cytotoxicity in the context of a 4-hour standard chromium release assay, not only because inhibition of its upregulation did not significantly affect cytotoxicity but also because there was considerably less FasL than granzyme B present in the CTLs. The possibility that FasL may be synthesised *de novo* and transported to the cell surface thereafter,

cannot be excluded, although this may be beyond the scope of the 4-hour chromium release cytotoxicity assays carried out here. However, a role for FasL/Fas in CTLs cytotoxicity remains unlikely as CD4+ CTLs and CD8+ CTLs (generated using allogeneic Fas deficient EBV-infected LCLs) not only efficiently lysed Fas-deficient target cells but their cytotoxicity was completely abrogated by CMA and EGTA (Yasukawa *et al*, 2000), in the same way as the CTLs described here.

Considerations of granzyme B and potentially granulysin delivery methods into target cells aside, it is clear that weekly stimulations with LCLs generated CTLs capable of specific cytotoxicity towards autologous LCLs. It is plausible that increased perforin expression by the CTLs may lead to increased cytotoxicity by facilitating the entry of more granzyme B into target cell, regardless of the mechanism of delivery.

6.4 CD4+ and CD8+ T-cells in the CTLs

The majority of CTLs generated in this project were predominantly CD8+ (82%), similar to those generated by Wilkie *et al* (2004), indicating that the stimulation of the CTLs with LCLs favoured their development over predominantly CD4+ CTLs. There was, however, always a small proportion of CD4+ T-cells present in CD8+ CTLs. This persistence may be intrinsically linked with the essential need for CD4+ T-cells by CD8+ T-cells to achieve a recall response following primary antigen exposure (Bevan, 2004). Whilst, CD8+ T-cells could effect a primary response in the absence of CD4+ T-cells, they could not respond to a second round of antigen exposure. As the CTLs generated here not only underwent primary and secondary antigen exposures but multiple weekly antigen exposures it is likely that the contribution of the CD4+ T-cells to the maintenance of the CTLs was critical. Furthermore CD4+ T-cells in the CTLs were also shown to express cytolytic molecules, indicating that they may play a dual "helper/killer" role in the CTLs. A critical role of CD4+ T-cells in CTLs is supported by observations made by Wilkie *et al* (2004), indicating that CTLs with a higher mean percentage of CD4+ T-cells did not have reduced cytotoxicity and in fact, these CTLs induced a better response in PTLD patients (Haque *et al*, manuscript in preparation).

6.5 Conclusions

This study shows that important phenotypic changes occur in the CTL cultures within the first four weeks and these changes persist later on in culture. This complements findings by Wilkie *et al* (2004), which show that the phenotype in terms of CD4+ and CD8+ was acquired within three to four weeks of culture. TCR spectratyping of the CTLs indicates that diversity of the repertoire was maintained over time. Further antigen specificity experiments would need to be carried out to assess how TCR repertoire diversity and usage might relate to antigen specificity and what the implications of TCR diversity versus uniformity are. Preliminary experiments carried out in this study show that CTLs are certainly specific for some antigens and not others. Characterising the antigen specificity of the CTLs may allow better matching of CTLs to PTLD tumours (based on antigen expression by the tumour). Finally, this study demonstrated that CTL cytotoxicity *in vitro*, and potentially *in vivo* (in CTLs which are already in clinical use), was perforin-dependent and that granzyme B was the most abundant cytolytic molecule. The CTLs may be effecting their role following a recently proposed mode of delivery whereby granzyme B and perforin dissociate from serglycin at the surface of the target cell, and enter the target cell by pinocytosis or endocytosis (mediated by binding to cell surface proteoglycan) subsequently leading to lysis of the endosome and apoptosis (Raja *et al*, 2005). This study also demonstrated that prolonged stimulation generated CTLs in which CD4+ T-cells and CD8+ T-cells differed from those found *in vivo* in terms of perforin expression but that this did not detrimentally affect cytotoxicity. Additionally, the CD4+ T-cells found in the CTLs were highly differentiated and expressed cytolytic molecules, albeit at significantly lower level than CD8+ T-cells (granzyme B only), indicating that CD4+ T-cells must play an active role in CTL-mediated cytotoxicity.

This study aimed to characterise CTLs in order to identify and increase understanding of features that may be adapted for improved clinical efficacy. Features which merit further investigation include modified culture methods that

could prolong culture period or enable specificity of the CTLs to be established before their viability is affected; the potential for manipulation of perforin expression to assess whether increased perforin levels may improve CTL cytotoxicity and also the question of the nature of the role of CD4+ T-cells during the course of the CTL culture – are they perhaps helper cells during phase 1 of growth and cytotoxic cells during phase 2 of growth?

6.6 Future developments

Since the beginning of this project, the generation of CTLs has progressed towards the development of CTLs specific to individual EBV antigens expressed in particular diseases, such as LMP-1 in HD and LMP-2 in both NPC and HD. Different methods have been used for the generation of CTLs. In one study autologous LCLs and PBMCs from NPC patients were used to generate CTLs (Straathof *et al*, 2005). In another study PBMCs were transduced with TCRs from LMP-2-specific clones giving rise to cytotoxic T-cells capable of lysing LCLs expressing LMP-2 (Jurgens *et al*, 2006).

Current investigations, in our laboratory, are concentrating on the generation of engineered T-cells. In these engineered T-cells, the parts of the α and β chains of the TCR which come in contact with the MHC/peptide complex are replaced with the Fab fragment of antibodies specific to EBV antigens. This system, overcomes HLA restriction, therefore considerably increasing the number of patients that may be treated with one CTL.

Chapter 7

7.1 Appendix A2

Decay of chromium-51

Adapted from data sheet provided by Amersham Biosciences.

Days before reference	**-16**	**-15**	**-14**	**-13**	**-12**	**-11**	**-10**	**-9**
	1.492	1.456	1.420	1.384	1.350	1.317	1.284	1.253
	-8	**-7**	**-6**	**-5**	**-4**	**-3**	**-2**	**-1**
	1.22	1.191	1.162	1.133	1.133	1.105	1.078	1.025
Days from reference	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	1.000	0.975	0.951	0.928	0.905	0.882	0.861	0.839
	8	**9**	**10**	**11**	**12**	**13**	**14**	**15**
	0.819	0.798	0.779	0.759	0.741	0.722	0.704	0.687
	16	**17**	**18**	**19**	**20**	**21**	**22**	**23**
	0.670	0.654	0.637	0.622	0.606	0.591	0.577	0.562
	24	**25**	**26**	**27**	**28**	**29**	**30**	**31**
	0.549	0.535	0.522	0.509	0.496	0.484	0.472	0.460

Table A2.1: Decay of ^{51}Cr

The volume of chromium required was calculated as follows:

$$\frac{\text{Activity required } (\mu Ci)}{\text{Activity factor given in the table above } (\mu Ci/ml)} = \text{volume of } ^{51}Cr \text{ required}$$

7.2 Appendix A3

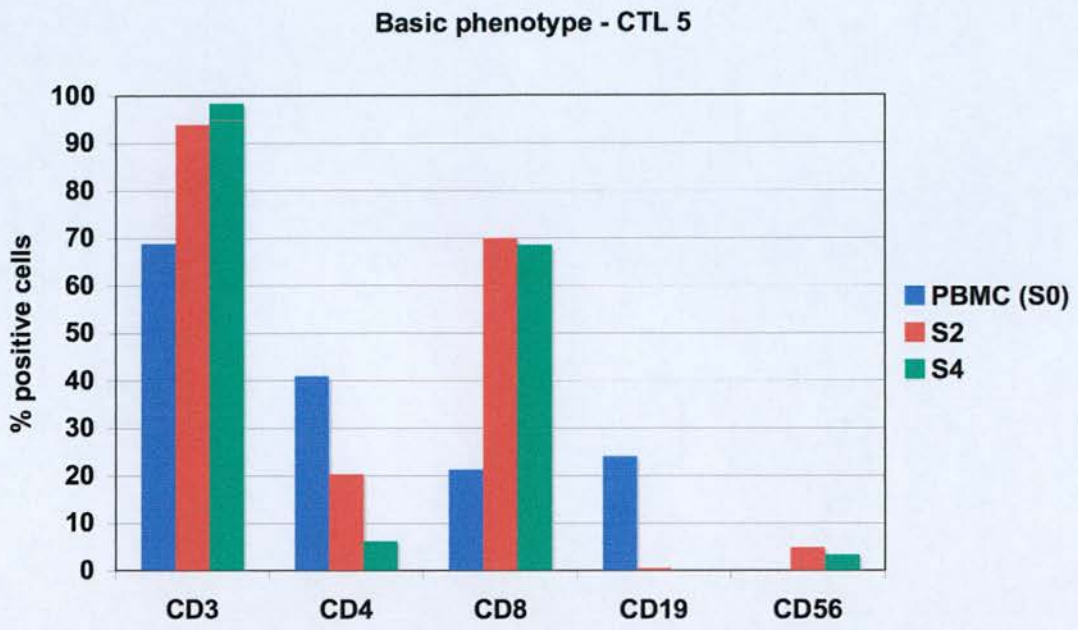


Figure A3.1: Basic phenotype of CTL 5.

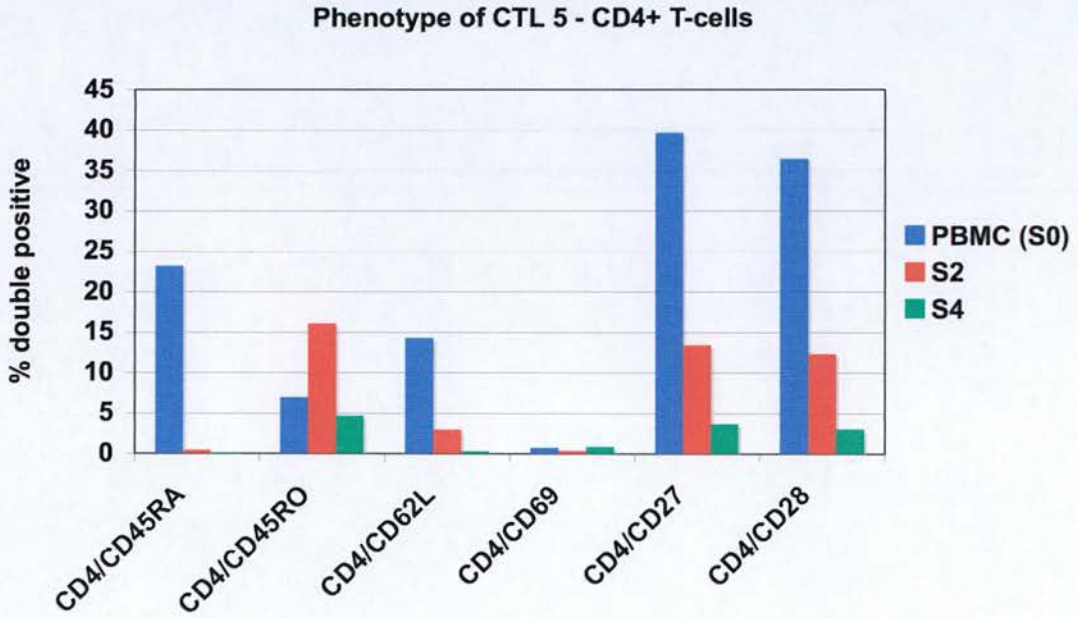


Figure A3.2: Phenotype of cells co-expressing CD4 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 5.

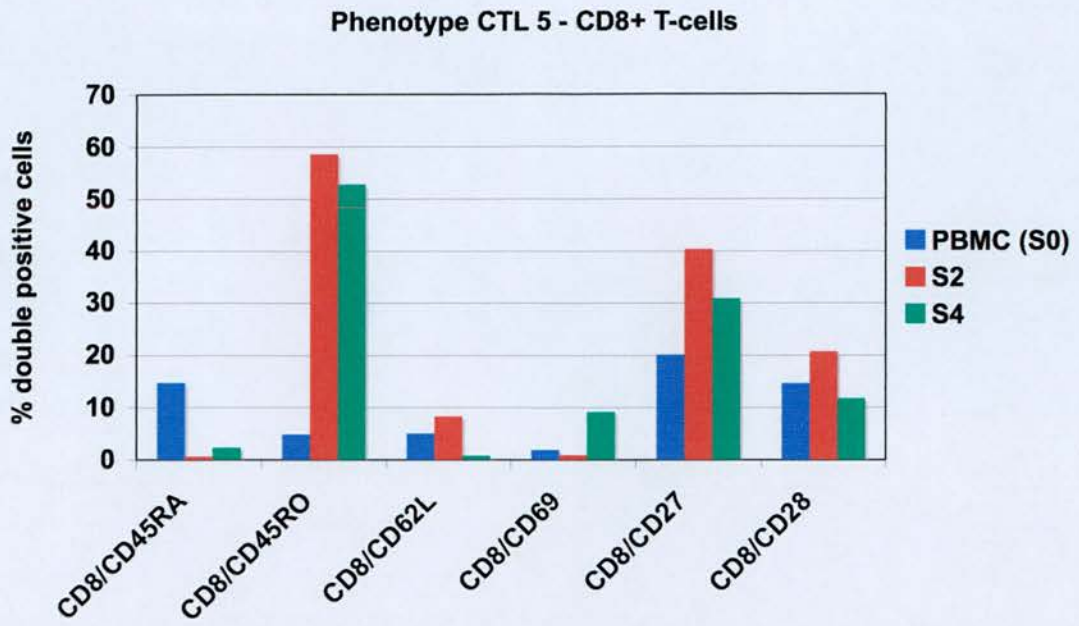


Figure A3.3: Phenotype of cells co-expressing CD8 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 5.

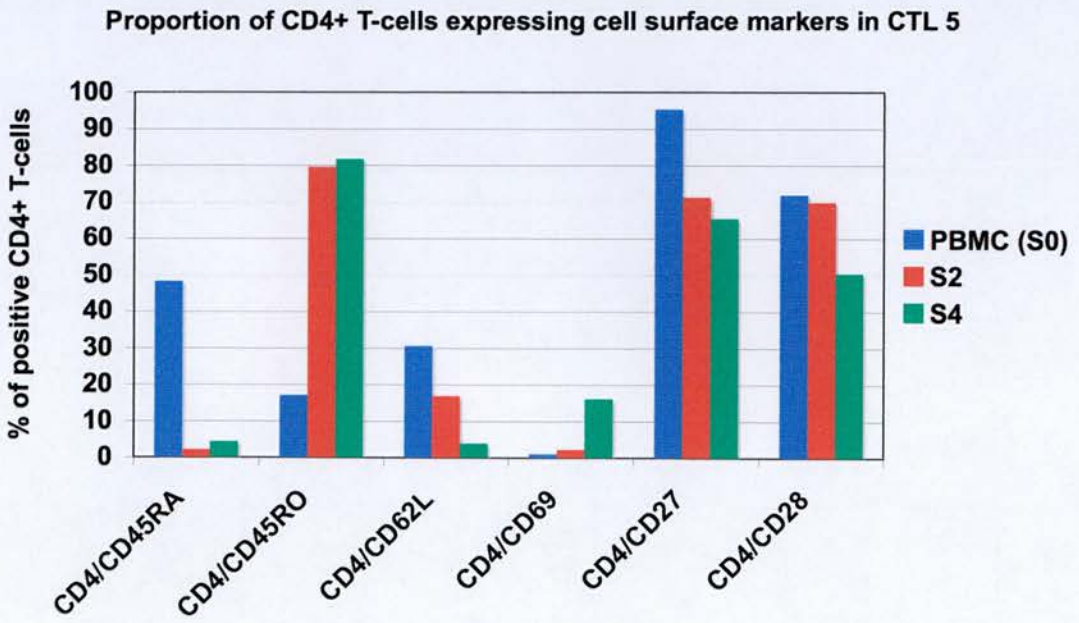


Figure A3.4: Proportion of CD4+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 5.

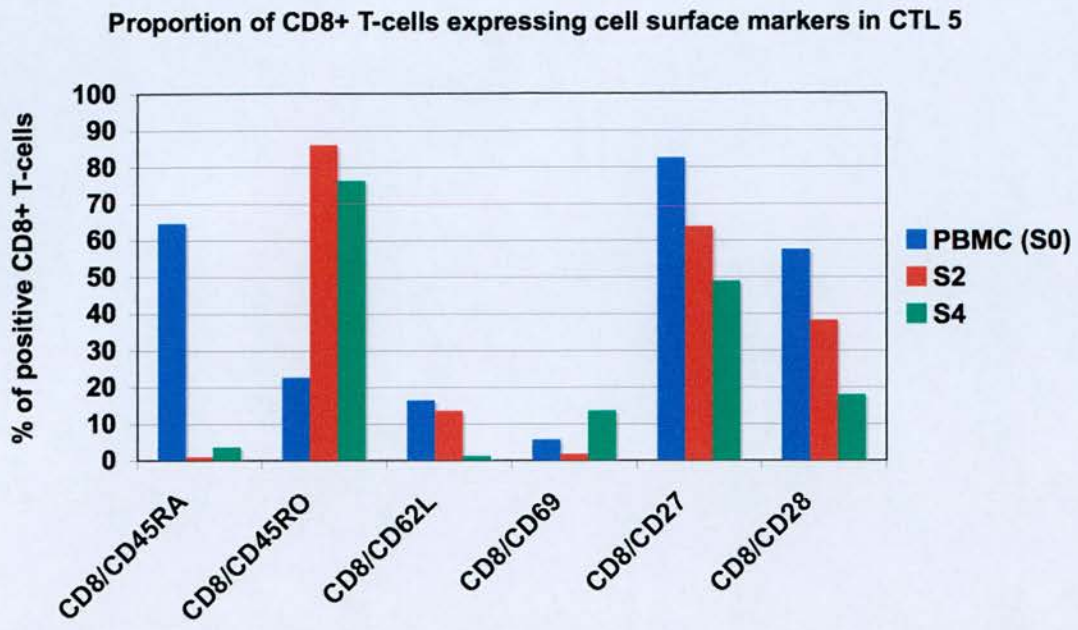


Figure A3.5: Proportion of CD8+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 5.

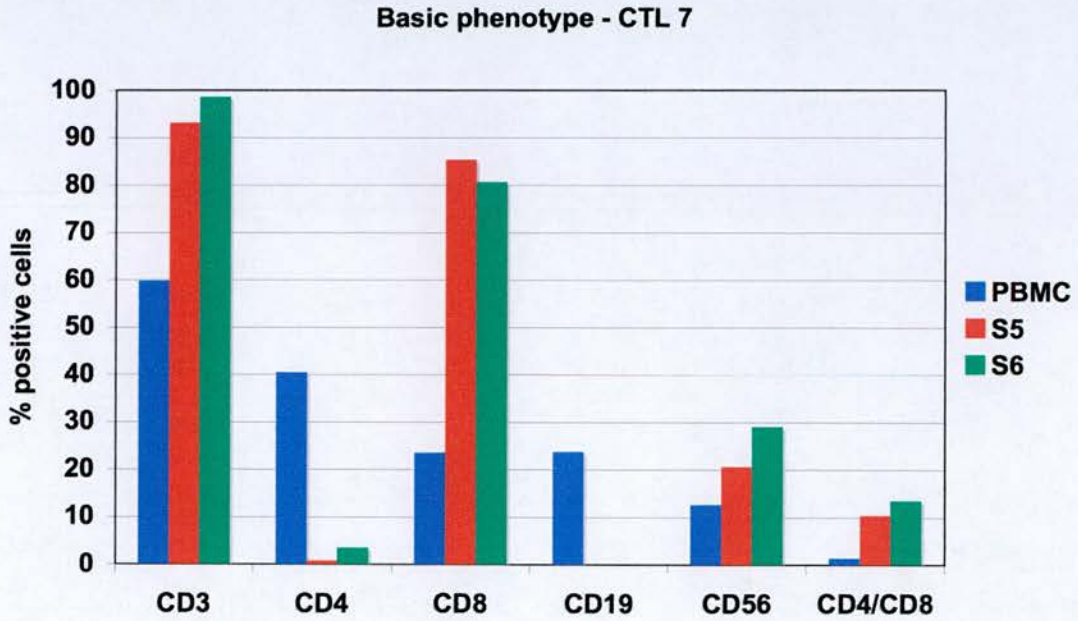


Figure A3.6: Basic phenotype of CTL 7.

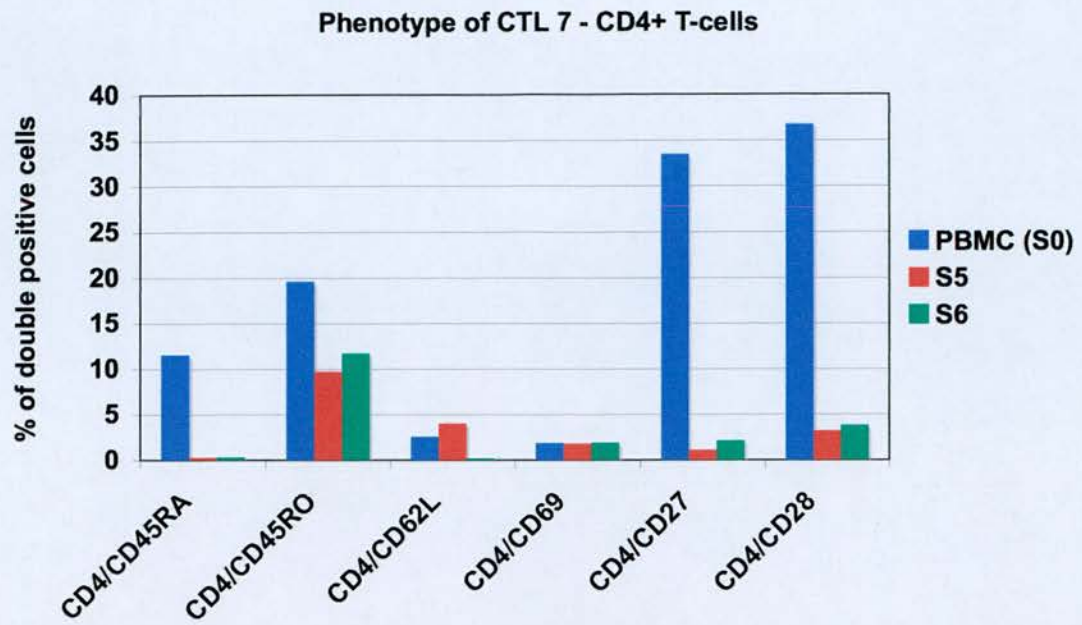


Figure A3.7: Phenotype of cells co-expressing CD4 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 7.

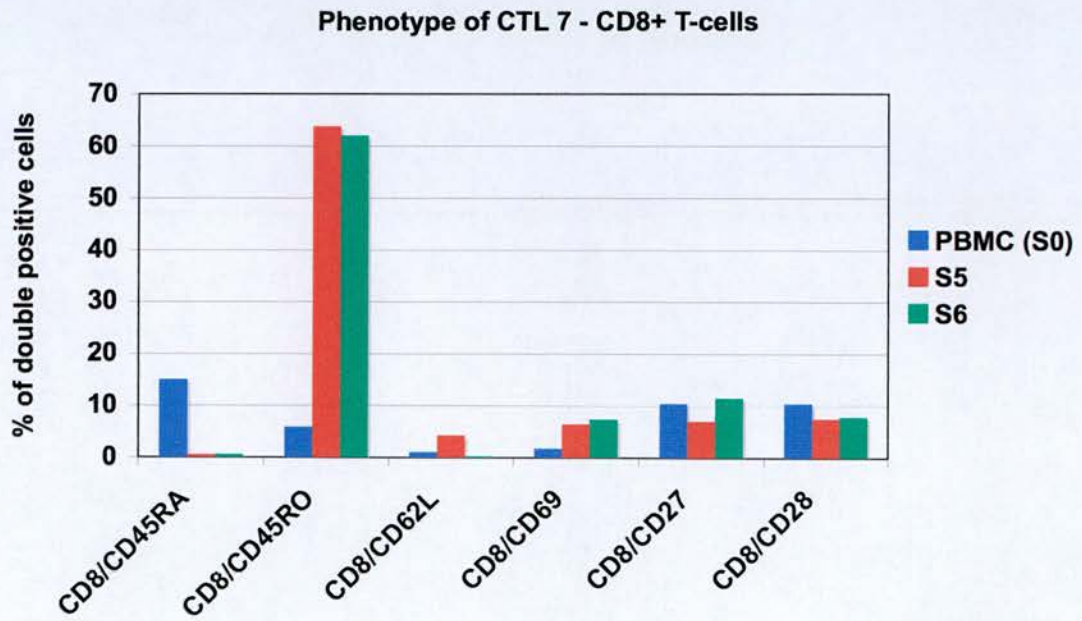


Figure A3.8: Phenotype of cells co-expressing CD8 and CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 7.

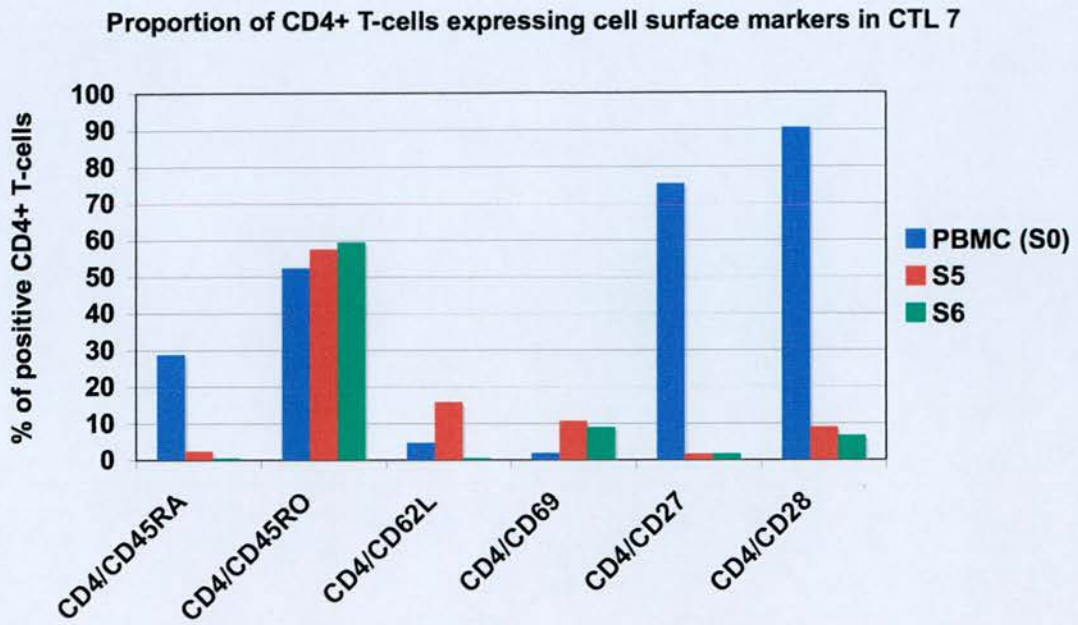


Figure A3.9: Proportion of CD4+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 7.

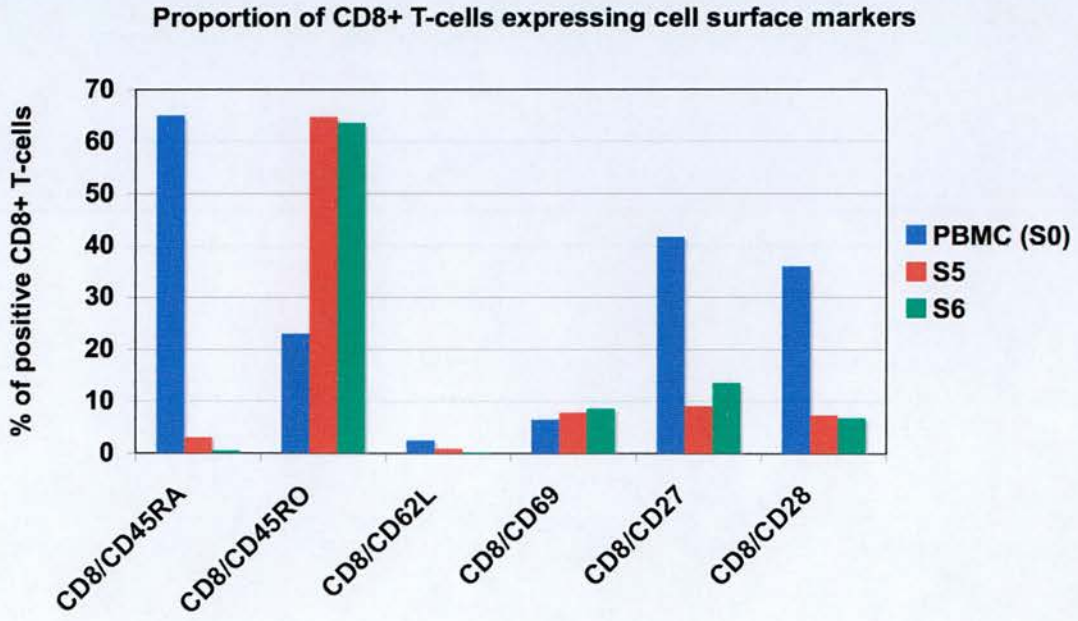


Figure A3.10: Proportion of CD8+ T-cells expressing CD45RA, CD45RO, CD62L, CD69, CD27 and CD28 in CTL 7.

7.3 Appendix A4

CTL 2	Nb of families used	Families used
S2	21	1,2,3,4,5,6,7,8,9,11,12,13,14,15,16,20,21,22,23,24,25
S3	18	1,2,3,4,6,7,8,11,12,13,14,15,16,17,20,21,22,24
S4	12	2,3,9,11,12,16,17,20,21,22,24
S5	22	1,2,3,4,5,6,7,8,9,11,12,13,14,15,16,17,18,20,21,22,23,24
Average nb of families used	18.25	All families were used.

Table A4.1: Vβ families used by CTL 2 during five weeks of culture with weekly stimulations with LCLs. Nb: number.

CTL 3	Nb of families used	Families used
PBMC	9	4,9,12,15,23,16,3,21,22
S3	16	1,2,3,4,5,6,7,8,9,12,13,14,15,11,17,23
S5	14	6,8,17,24,11,13,2,3,5,9,12,15,21,22
Average nb of families used	13	All families were used except families Vβ-18, -20, -24 and -25.

Table A4.2: Vβ families used by CTL 3 during five weeks of culture with weekly stimulations with LCLs. Nb: number.

CTL 13	Nb of families used	Families used
S4	9	3,6,7,9,12,13,20,21,22
S5	16	2,3,4,5,6,7,8,9,12,13,15,17,20,21,22,24
S7	18	1,2,3,4,5,6,8,9,11,12,13,16,17,18,20,21,22,23
S8	22	2,3,4,5,6,7,8,9,11,12,13,14,15,16,17,18,20,21,22,23,24
Average nb of families used	16.25	All families were used except family Vβ-25.

Table A4.3: Vβ families used by CTL 13 during eight weeks of culture with weekly stimulations with LCLs. Nb: number.

CTL 4	Nb of families used	Families used
PBMC	18	2,3,4,5,6,8,9,11,12,13,14,15,16,17,21,22,23,24
S4	17	1,2,3,4,5,6,7,8,9,12,13,14,15,17,21,23,24
S5	19	1,2,3,4,5,6,7,8,9,12,13,14,15,16,17,18,21,22,23
S6	14	2,3,4,5,6,7,8,12,13,14,15,16,21,22
S7	16	1,2,3,4,5,6,8,9,12,13,14,15,16,21,22,23
S8	16	1,2,3,4,5,8,9,11,12,13,14,17,21,22,23,24
S9	12	2,3,4,5,6,8,9,12,13,14,21,23
Average nb of families used	16	All families were used except families Vβ-20 and Vβ-25.

Table A4.4: Vβ families used by CTL 4 during nine weeks of culture with weekly stimulations with LCLs. Nb: number.

CTL 5	Nb of families used	Families used
PBMC	18	2,3,4,5,6,8,9,11,12,13,14,15,16,17,21,22,23,24
S1	8	2,3,4,7,9,11,17,23
S2	15	1,2,3,4,5,7,8,9,15,16,17,22,23,24,25
S3	14	1,2,3,5,6,7,8,14,16,17,21,22,24,25
S4	18	1,3,4,5,6,7,8,9,11,12,13,16,17,18,22,23,24,25
S5	14	3,5,7,8,9,13,14,15,16,17,21,22,23,25
S6	16	1,2,3,4,5,6,7,8,9,13,14,15,16,17,23,24
Average nb of families used	14.7	All families were used except family Vβ-20.

Table A4.5: Vβ families used by CTL 5 during six weeks of culture with weekly stimulations with LCLs. Nb: number.

CTL 7	Nb of families used	Families used
PBMC	15	2,3,4,5,6,8,9,11,15,16,20,21,22,23,24
S1	13	1,2,3,4,5,6,7,8,9,12,14,15,22
S2	20	1,2,3,4,5,6,8,9,11,12,13,14,15,16,17,18,20,21,23,24
S3	20	1,2,3,4,5,6,7,8,9,12,13,14,15,16,17,18,20,21,22,23
S4	17	1,3,4,5,6,7,8,9,11,14,15,16,17,18,21,22,24
S5	20	1,2,3,4,6,7,8,9,12,13,14,15,16,17,18,20,21,22,23,24
S6	20	1,2,3,4,5,6,7,8,9,11,12,13,15,16,17,20,21,22,23,24
S7	19	1,2,3,4,5,6,7,8,9,11,12,13,14,16,20,21,22,23,24
S8	19	1,2,3,4,5,6,7,8,9,13,14,15,16,17,18,22,23,24,25
S15	17	3,4,6,9,11,12,13,14,15,16,17,18,20,21,22,23,25
Average nb of families used	18	All families were used.

Table A4.6: Vβ families used by CTL 7 during 15 weeks of culture with weekly stimulations with LCLs. Nb: number.

CTL 12	Nb of families used	Families used
S2	22	1,2,3,4,5,6,7,8,9,11,12,13,14,15,16,17,18,20,21,22,23,24
S4	21	1,2,3,4,5,6,7,8,9,11,12,13,14,15,16,17,20,21,22,23,24
S5	19	1,2,3,4,5,6,7,8,9,12,13,14,15,16,17,20,21,22,25
S6	12	2,3,4,5,7,12,14,15,16,21,22,23
S7	13	2,3,4,5,6,8,9,12,14,16,21,22,23
Average nb of families used	17.4	All families were used.

Table A4.7: Vβ families used by CTL 12 during seven weeks of culture with weekly stimulations with LCLs. Nb: number.

7.4 Appendix A5

E:T 20:1	No inhibitor	10nM CMA	40μM BFA	3mM EGTA
CTL 5	65.7%	18.9%	78.6%	1.0%
CTL 7	77.1%	50.9%	50.6%	14.2%
CTL 9	56.7%	18.3%	52.7%	5.3%
CTL 15	29.0%	1.1%	20.4%	1.6%
CTL 16	46.5%	1.7%	35.3%	1.2%
CTL 17	50.8%	0.4%	42.9%	0.2%
CTL 19	40.9%	0.1%	27.4%	0.1%
CTL 20	33.3%	0%	23.2%	0%
CTL 21	25.9%	0%	25.8%	0.9%
CTL 22	18.1%	2.0%	15.3%	0.8%
CTL 23	16.8%	1.1%	14.1%	1.5%
CTL 24	24.5%	0.3%	13.8%	2.7%
Mean % cytotoxicity	40.5%	7.5%	33.3%	2.4%
Median % cytotoxicity	37.1%	1.1%	26.6%	1.1%

Table A5.1: Percentage of autologous LCL lysis without inhibitor, with CMA, BFA and EGTA at E:T 20:1. n=12 CTLs.

E:T 10:1	No inhibitor	10nM CMA	40μM BFA	3mM EGTA
CTL 15	29.4%	1.8%	15.2%	0.1%
CTL 16	36.6%	0%	29.9%	0.6%
CTL 17	39.8%	0%	34.4%	0%
CTL 19	34.3%	0%	25.4%	0%
CTL 20	29.3%	0%	16.3%	0%
CTL 21	21.9%	0%	24.0%	0%
CTL 22	12.3%	1.4%	11.7%	0%
CTL 23	13.4%	0.3%	9.8%	0.5%
CTL 24	12.9%	0.8%	8.3%	0%
Mean % cytotoxicity	25.6%	0.5%	19.5%	0.1%
Median % cytotoxicity	29.3%	0.0%	16.3%	0%

Table A5.2: Percentage of autologous LCL lysis without inhibitor, with CMA, BFA and EGTA at E:T 10:1. n=9 CTLs.

E:T 5:1	No inhibitor	10nM CMA	40μM BFA	3mM EGTA
CTL 15	20.3%	1.6%	7.2%	0.4%
CTL 16	29.6%	1.8%	24.6%	0%
CTL 17	32%	0%	25%	0%
CTL 19	33%	0.9%	24.1%	0.7%
CTL 20	23.4%	0%	15%	0%
CTL 21	15.1%	0%	15.4%	2.1%
CTL 22	11.2%	0.4%	8.9%	0%
CTL 23	8.8%	0.6%	6.9%	0%
CTL 24	8.8%	0.2%	2.5%	0.8%
Mean % cytotoxicity	20.2%	0.6%	14.4%	0.4%
Median % cytotoxicity	20.3%	0.4%	15%	0%

Table A5.3: Percentage of autologous LCL lysis without inhibitor, with CMA, BFA and EGTA at E:T 5:1. n=9 CTLs.

% reduction in autologous LCL lysis at E:T 10:1	Induced by 10nM CMA	Induced by 40μM BFA	Induced by 3mM EGTA
CTLs			
CTL 15	93.8%	15.2%	99.8%
CTL 16	100%	18.4%	98.5%
CTL 17	100%	13.8%	100%
CTL 19	100%	26.0%	100%
CTL 20	100%	44.3%	100%
CTL 21	100%	0%	100%
CTL 22	88.9%	4.7%	100%
CTL 23	97.6%	26.7%	96.5%
CTL 24	93.9%	35.4%	100%
Mean % reduction in cytotoxicity	97.1%	19.4%	99.4%
Median % reduction in cytotoxicity	100%	18.4%	100%

Table A5.4: Percentage reduction in autologous LCL lysis induced by the CMA, BFA and EGTA at E:T 10:1. n=9 CTLs.

% reduction in autologous LCL lysis at E:T 5:1	Induced by 10nM CMA	Induced by 40μM BFA	Induced by 3mM EGTA
CTLs			
CTL 15	92%	7.2%	98%
CTL 16	94.1%	17.1%	100%
CTL 17	100%	21.8%	100%
CTL 19	97.3%	26.8%	98%
CTL 20	100%	36.1%	100%
CTL 21	100%	0%	100%
CTL 22	96.2%	19.9%	100%
CTL 23	93.3%	21.1%	100%
CTL 24	97.5%	71.6%	90.9%
Mean % reduction in cytotoxicity	96.7%	24.4%	98.5%
Median % reduction in cytotoxicity	97.3%	21.1%	100%

Table A5.5: Percentage reduction in autologous LCL lysis induced by the CMA, BFA and EGTA at E:T 5:1. n=9 CTLs.

CTLs	Perforin MFI (AU)	Granzyme B MFI (AU)	FasL MFI (AU)
CTL 5	0.18	31.44	1.32
CTL 7	0.16	30.95	1.68
CTL 9	0.07	83.7	1.05
CTL 15	2.34	46.91	8.7
CTL 16	1.8	23.87	4.75
CTL 17	1.53	39.95	5.26
CTL 19	1.6	45.46	6.57
CTL 20	3.33	69.43	8.65
CTL 21	2.07	59.3	5.27
CTL 22	1.23	30.33	5.37
CTL 23	1.24	58.02	5.36
CTL 24	1.46	NA	4.64

Table A5.6: MFI of perforin, granzyme B and FasL in all CTLs. AU: arbitrary units. NA: not available.

CTLs	CD4+ T-cells (MFI in AU)			CD8+ T-cells (MFI in AU)		
	Perforin	Granzyme B	FasL	Perforin	Granzyme B	FasL
CTL 5	0.19	7.32	1.17	0.39	30.80	2.01
CTL 7	0	16.52	0.52	0.17	45.15	1.26
CTL 9	0.42	38.24	2.15	0	43.29	0.74
CTL 15	1.12	25.23	6.52	2.41	48.56	8.84
CTL 16	0.54	10.54	3.26	1.92	25.4	3.26
CTL 17	0.73	32.83	3.9	1.67	41.3	5.59
CTL 19	0.49	11.8	4.61	1.7	48.91	6.74
CTL 20	3.78	40.04	6.38	3.35	69.79	8.71
CTL 21	1.27	36.64	3.8	2.15	60.53	5.44
CTL 22	0.92	9.77	4.72	1.2	32.4	5.43
CTL 23	1.22	22.76	4.74	1.23	60.3	5.4
CTL 24	1.55	NA	4.64	1.45	NA	4.64
Median MFI	0.66	22.76	4.68	1.22	48.91	5.52
Mean MFI	1.35	22.91	5.59	1.11	68.23	5.76

Table A5.7: MFI of perforin, granzyme B and FasL in CD4+ T-cells and CD8+ T-cells. AU: arbitrary units. NA: not available.

CTLs	% of perforin positive cells	% of granzyme B positive cells	% of FasL positive cells
CTL 5	0.7%	68.2%	0.5%
CTL 7	1.1%	71.6%	2.9%
CTL 9	1.1%	84.7%	1.5%
CTL 15	2.1%	89.6%	27.8%
CTL 16	0.1%	81.3%	2.9%
CTL 17	0.3%	96.7%	5.9%
CTL 19	0.2%	92%	8.2%
CTL 20	3.1%	99.2%	26.5%
CTL 21	3.3%	93.3%	27.4%
CTL 22	0.5%	87.1%	11.8%
CTL 23	0.4%	98.4%	9.8%
CTL 24	0.4%	NA	5.8%

Table A5.8: Proportion of cells positive for perforin, granzyme B and FasL expression in CTLs.

CTLs	CD4+ T-cells			CD8+ T-cells		
	% Per	% GrB	% FasL	% Per	% GrB	% FasL
CTL 5	3.9%	23.9%	2.6%	0.4%	72.0%	1.7%
CTL 7	0.7%	50.8%	2.9%	2%	73.2%	3.6%
CTL 9	11.3%	65.6%	13.2%	1.3%	80.1%	1.8%
CTL 15	1.5%	70.8%	7.5%	2.5%	89.3%	15.0%
CTL 16	0.4%	33.5%	3.3%	0.1%	85.1%	4.3%
CTL 17	0.8%	91.5%	2.2%	0.1%	91.5%	6.5%
CTL 19	0.7%	15.1%	5.2%	0.1%	78.2%	8.3%
CTL 20	13.9%	69.6%	14.7%	3.5%	97.7%	14.4%
CTL 21	3.3%	87.3%	16.1%	3.4%	93.5%	28.5%
CTL 22	1.5%	22.5%	9.1%	0.3%	77.4%	11.5%
CTL 23	5.4%	69.2%	18.5%	0.1%	96.2%	9.7%
CTL 24	0.2%	NA	10.2%	15%	NA	5.6%

Table A5.9: Percentage of CD4+ T-cells and CD8+ T-cells in all CTLs expressing perforin, granzyme B and FasL. Per: perforin and GrB: granzyme B. NA: not available.

CTLs	% CD4+ T-cells	% CD8+ T-cells	% CD4-/CD8- cells	% CD4+/CD8+ T-cells
CTL 5	20.3%	77%	ND	ND
CTL 7	51.8%	45.5%	ND	ND
CTL 9	9.5%	89.7%	ND	ND
CTL 15	2.6%	94.3%	2.2%	0.9%
CTL 16	6.5%	91.8%	0.9%	0.8%
CTL 17	13.5%	83.7%	2.3%	0.5%
CTL 19	5.6%	91.7%	2%	0.7%
CTL 20	0.4%	98.1%	1.4%	0.1%
CTL 21	2.5%	92%	4.9%	0.6%
CTL 22	5.1%	90.4%	3.1%	1.4%
CTL 23	3.2%	94.8%	1.3%	0.7%
CTL 24	1.3%	96.2%	1.6%	0.9%

Table A5.10: Proportion of CD4+ T-cells and CD8+ T-cells in the CTLs.

Chapter 8

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